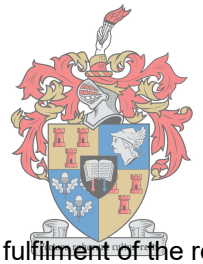
Combinatorial treatments of tamoxifen with SM6Met, a selective estrogen receptor subtype modulator (SERSM), from *Cyclopia subternata* are superior to current endocrine treatments in breast cancer cell models

by

Lorinda van Dyk



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Declaration

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Summary

Globally and in South Africa breast cancer is the most frequent malignancy amongst women. Most breast cancers are estrogen receptor (ER) positive and requires estrogen for growth and metastasis. Adverse side-effects associated with, and resistance to, the current standard of care (SOC) hormone therapies that target estrogen signalling, like tamoxifen, a selective estrogen modulator (SERM), and fulvestrant, a selective estrogen receptor down-regulator (SERD), have driven the recent development of using natural compounds or extracts as novel therapies, either alone or in combination with conventional chemotherapeutic agents, for the treatment and/or prevention of breast cancer. Previous work from our laboratory has suggested that a sequential methanol extract, SM6Met, prepared from the indigenous fynbos plant, *Cyclopia subternata* (honey bush), has several properties that may make it an effective chemopreventative and/or chemotherapeutic agent for breast cancer. The current study investigated the ability of SM6Met to prevent or treat breast cancer either as monotherapy or in combination with 4-OH-Tam (the active metabolite of tamoxifen) by evaluating its effects on the processes required for the development and progression of breast cancer such as proliferation, migration, invasion and colony formation. Firstly, I validated previous findings, characterizing SM6Met as a selective estrogen receptor subtype modulator (SERSM) that behaves as an ERα antagonist and ERβ agonist, which is able to inhibit estrogen-induced breast cancer cell proliferation. Importantly, I show that although SM6Met as monotherapy could not compete, in terms of efficacy or potency, with current SOC therapies like 4-OH-Tam and fulvestrant, with regard to inhibiting breast cancer cell proliferation, SM6Met showed potential in targeting two pro-metastatic processes, invasion and colony formation, to an extent equal to, if not greater than the SOC therapies and thus has the potential to be developed into a phytoestrogenic nutraceutical that can be beneficial in the prevention and treatment of breast cancer and metastasis. Secondly, I show, for the first time, that the effects of SM6Met could be replicated and enhanced by combining an ERα selective antagonist (MPP) and an ERβ selective agonist (liquiritigenin), thus validating the concept that a treatment with these ideal ER subtype selective properties may be more beneficial for the treatment and prevention of breast cancer and metastasis than current SOC therapies. Thirdly, I show, for the first time, that the combination therapy of 4-OH-Tam and SM6Met produced a strong synergistic effect in terms of antagonizing breast cancer cell proliferation and that a 20 times lower dose of 4-OH-Tam in combination with SM6Met is required to produce the same inhibitory effect on cell proliferation as 4-OH-Tam alone. Moreover, the best combination ratio (20:1) of SM6Met with 4-OH-Tam displayed greater anti-metastatic potential than the extract or the SOC therapies alone, suggesting that SM6Met together with 4-OH-Tam could be a viable drug combination for not only delaying resistance and ameliorating the negative side effects associated with current SOC therapies, like tamoxifen, but could also provide a novel, more affordable therapeutic alternative for treating or preventing breast cancer metastasis.

Opsomming

Wêreldwyd en in Suid-Afrika is borskanker die mees algemene kanker onder vroue. Meeste borskankers is estrogeen reseptor (ER) positief en benodig estrogeen vir groei en metastase. Die verskeideneid newe-effekte wat geassosieer is met, asook weerstand teen, die huidige standaard gebruikte hormoon terapie wat estrogeen-sein teiken, soos tamoxifen, 'n selektiewe estrogeen reseptor modulator (SERM) en fulvestrant, 'n selektiewe estrogeen reseptor af-regulator (SERD), het die onlangse ontwikkeling van die gebruik van natuurlike verbindings of ekstrakte as nuwe terapieë gedryf, hetsy alleen of in kombinasie met konvensionele chemoterapeutiese middels, vir die behandeling en/of voorkoming van borskanker. Vorige werk uit ons laboratorium het getoon dat 'n opeenvolgende metanol ekstrak, SM6Met, wat vanaf die inheemse fynbosplant, *Cyclopia* subternata (heuningbos) voorberei is, verskeie eienskappe het wat dit 'n effektiewe chemovoorkomings en/of chemoterapeutiese middel vir borskanker kan maak. Die huidige studie het die potensiaal van SM6Met ondersoek om borskanker te voorkom of te behandel as enkelterapie of in kombinasie met 4-OH-Tam (die aktiewe metaboliet van tamoxifen) deur die effekte daarvan te evalueer op die prosesse wat nodig is vir die ontwikkeling en progressie van borskanker soos proliferasie, migrasie, indringing en kolonie vorming. Eerstens, bevestig ons vorige bevindinge, wat SM6Met kenmerk as 'n selektiewe estrogeen reseptor subtipe modulator (SERSM) wat optree as 'n ERα-antagonis en ERβ-agonis, wat estrogeen-geïnduseerde borskanker-proliferasie kan inhibeer. Verder, wys ons dat, alhoewel SM6Met as monoterapie, met betrekking tot die inhibering van borskanker-proliferasie, nie in terme van effektiwiteit of sterkte kon meeding met huidige standaard gebruikte hormoon terapieë soos 4-OH-Tam en fulvestrant nie, wys SM6Met potensiaal om twee pro-metastatiese prosesse, indringing en kolonie vorming, te inhibeer tot 'n mate gelyk aan, indien nie groter as die standaard gebruikte hormoon terapieë nie, en sodoende het SM6Met die potensiaal om as fitoëstrogeniese neutraseutiese middel ontwikkel te word wat voordelig kan wees in die voorkoming en behandeling van borskanker en metastase. Tweedens wys ons, vir die eerste keer, dat die effekte van SM6Met gerekonstruktureer kan word deur 'n ERα selektiewe antagonis (MPP) en 'n ERβ selektiewe agonis (liquiritigenin) saam te kombineer, wat die konsep bevestig dat 'n behandeling met hierdie ideale ER subtipe selektiewe eienskappe meer voordelig kan wees vir die behandeling en voorkoming van borskanker en metastase as huidige standaard hormoon terapieë. In die derde plek wys ons, vir die eerste keer, dat die kombinasie terapie van 4-OH-Tam en SM6Met 'n sterk sinergistiese effek toon in terme van inhibisie van borskanker-proliferasie en dat 'n 20 keer laer dosis 4-OH-Tam in kombinasie met SM6Met nodig is om dieselfde vlak van inhibisie as 4-OH-Tam alleen te produseer. Daarbenewens, het die beste kombinasie verhouding (20: 1) van SM6Met tot 4-OH-Tam groter anti-metastatiese potensiaal as die ekstrak alleen of die huidige standaard gebruikte hormoon terapieë alleen getoon, wat daarop dui dat SM6Met tesame met 4-OH-Tam 'n lewensvatbare geneesmiddelkombinasie kan wees, om nie net weerstand te vertraag en die negatiewe newe-effekte wat met huidige standaard gebruikte hormoon terapieë, soos tamoxifen, geassosieer word te verlaag nie, maar kan ook 'n nuwe, meer bekostigbare terapeutiese alternatief vir die behandeling of voorkoming van borskankermetastase voorsien.

I would like to dedicate this dissertation to my parents, Amanda and Lourens van Dyk. Thank you for supporting and believing in me and giving me the opportunities I needed to be where I am today.

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Alphabetical list of abbreviations

4-OH-Tam	-	(2)-4-hydroxytamoxifen
AF-1	-	Activation function-1
AI	-	Aromatase inhibitor
AIDS	-	Acquired Immune Deficiency Syndrome
ANOVA	-	Analysis of variance
CDK	-	Cyclin-dependent kinase
DBD	-	DNA binding domain
D-box	-	Dimerization box
DMEM	-	Dulbecco's modified Eagle's medium
DMSO	-	Dimethyl sulfoxide
DNA	-	Deoxyribonucleic acid
DS	-	Double stripped
E1	-	Estrone
E2	-	17-β-estradiol
E3	-	Estriol
EC50	-	Half maximal effective concentration
Efficacy	-	Maximal response induced
EGFR	-	Epidermal growth factor receptor
ER	-	Estrogen receptor
ERE	-	Estrogen response element
ERα	-	Estrogen receptor alpha
ERβ	-	Estrogen receptor beta
EtOH	-	Absolute ethanol
FCS	-	Fetal calf serum
Ful	-	Fulvestrant
GAPDH	-	Glyceraldehyde 3-phosphate dehydrogenase
hERα	-	Human estrogen receptor alpha
hERβ	-	Human estrogen receptor beta
HI	-	Heat inactivated

kDa	-	Kilo Dalton
LBD	-	Ligand binding domain
Liq	-	Liquiritigenin
Luc	-	Luciferase
MPP	-	Methyl-piperidino-pyrazole
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFκB	-	Nuclear factor-kappa B
P-box	-	proximal box
PBS	-	Phosphate-buffered saline
Potency	-	EC_{50}
SEM	-	Standard error of the mean
SERD	-	Selective estrogen receptor degrader
SERM	-	Selective estrogen receptor modulator
SERSM	-	Selective estrogen receptor subtype modulator
SM6Met	-	Sequential methanol extract of *Cyclopia subternata* harvesting M6
SOC	-	Standard of Care
SSH	-	SERM/SERD Hybrid

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Chapter 1

Introduction

In recent years, breast cancer has come to be the most common and deadly forms of cancer amongst women globally, and although statistics differ between populations, age and ethnic groups, women from developing countries such as South Africa present with an overall poorer prognosis regarding breast cancer survival than those from well-developed countries (1–7). It seems reasonable to postulate that the high cost of modern chemotherapeutic drugs have played a major role in the overall poorer prognosis. Apart from the high costs, endocrine resistance and negative side effects have also proved to be significant issues in breast cancer treatment (8). According to the national cancer registry, breast cancer incidence has increased by 72.5% amongst all women in South Africa from 2000 to 2014 (9). The high incidence of breast cancer, its contribution to cancer-related deaths and drawbacks associated with endocrine therapies have primarily driven the more recent development of novel therapies for breast cancer prevention and treatment that act through multiple mechanisms, in addition to new strategies like combined therapies, many of which notably include the use of more natural products, such as tea leaf extracts, in combination with conventional chemotherapeutic agents (8, 10, 11). Many such combined therapies have been shown to produce synergistic anti-cancer effects with tamoxifen as the conventional chemotherapeutic agent (10, 11).

Tamoxifen was the first selective estrogen receptor modulator (SERMs) to be approved for breast cancer prevention and treatment and its major molecular target, the estrogen receptor (ER), is overexpressed in 70% of breast tumours (12). The ER has been identified as a viable therapeutic target to overcome endocrine resistance, which suggests that the addition of another ER-targeted anti-cancer agent in combination with current standard of care (SOC) endocrine therapies, like tamoxifen, could prove effective for overcoming breast tumour resistance to tamoxifen (13). The ER consists of an alpha (α) and beta (β) subtype, where ERα subtype signalling has been associated with sustained breast cancer cell proliferation (and other cancer hallmarks) and ERβ subtype signalling has been linked to amelioration of ERα's cancer promoting effects (14–22). Therefore, compounds that mediate their effects primarily via ERβ, like the natural phytoestrogenic compounds, genistein (soy) and biochanin A (red clover), are receiving greater interest for the development of novel, cost effective and affordable therapies for breast cancer prevention and treatment (23–26).

Numerous studies have shown a strong correlation between the consumption of plant-based diets or plant extracts and the prevention and overall reduction of cancer, including breast cancer (27–33). Natural compounds and extracts elicit their anti-cancer effects by targeting multiple pathways involved in the process of breast carcinogenesis. Moreover, these natural compounds and extracts have been shown to not only ameliorate side-effects, such as nausea, fatigue, anaemia and mucositis, arising from currently used chemotherapy or other breast cancer treatments (34), but have also shown synergistic action and/or

enhancement of effectiveness when used in combination with other chemotherapeutic or endocrine therapy agents (35). These beneficial outcomes have shifted pharmacological interest from highly selective single targeted therapies to compounds or extracts that may inhibit multiple molecular targets in breast cancer. Moreover, the distinct physiological roles of the ER subtypes, ERα and ERβ, warrant further investigation into compounds or extracts that may act as ERα antagonists and ERβ agonists as a potential novel treatment strategy for breast cancer.

The current study thus expands on previous studies to evaluate the potential of SM6Met, a sequential methanol extract of the indigenous fynbos plant, *Cyclopia subternata* or otherwise known as honey bush, as an alternative for breast cancer treatment and prevention in comparison to SOC endocrine therapies, such as tamoxifen. SM6Met was first identified in our laboratory as having an estrogenic potency comparable to many commercial phytoestrogenic nutraceuticals (20, 21, 36). More recent studies revealed that SM6Met selectively targets the ER subtypes, by acting as an ERα antagonist and ERβ agonist (20, 21, 37, 38) and inhibits estrogen induced breast cancer cell proliferation *in vitro* (21). Furthermore, *in vivo* studies, showed that SM6Met delays ERα induced rat uterine growth (21) and reduces *N*-Methyl-*N*-nitrosourea (MNU)-induced and LA7 cell-induced rat mammary tumours (39, 40).

The main focus of the current study was to explore the physiological implications of the dual activity (multi-targeting) of the selective estrogen receptor subtype modulator (SERSM), SM6Met, on the processes required for the development and progression of breast cancer such as proliferation, migration, invasion and colony formation, either as monotherapy or in combination with current SOC therapies, such as tamoxifen. In chapter 3 and 4, the following aims will be addressed:

1. To investigate, with regard to their effects on human breast cancer cell proliferation,

   Objectives:

   a. To compare SM6Met to SOC therapies, like 4-OH-Tam (the active metabolite of tamoxifen) and fulvestrant

   b. To replicate the properties of SM6Met can by combining an ERα antagonist (MPP) with an ERβ agonist (liquiritigenin)

   c. To evaluate the synergistic potential of SM6Met in combination with 4-OH-Tam and how the combination of 4-OH-Tam with SM6Met compares to 4-OH-Tam alone

2. To investigate, with regard to their effects on human breast cancer cell cycle regulation as well as metastatic potential,

   Objectives:

   a. To compare SM6Met to SOC therapies, like 4-OH-Tam and fulvestrant

b. To replicate the properties of SM6Met can by combining an ERα antagonist (MPP) with an ERβ agonist (liquiritigenin)

c. To evaluate the synergistic potential of SM6Met in combination with 4-OH-Tam and how the combination of 4-OH-Tam with SM6Met compares to 4-OH-Tam alone

This dissertation consists of five chapters. The literature review in Chapter 2 will cover the process of carcinogenesis with the focus on breast cancer, the role of estrogen in breast cancer development, current treatments for breast cancer that target the ER, drawbacks of these therapies and alternative strategies to overcome breast cancer. Chapter 3 (addressing aim 1) and Chapter 4 (addressing aim 2), are written in manuscript format, where each chapter contains an introduction, methods, results and discussion section and therefore some repetition will inevitably occur. In the final chapter (Chapter 5), the results from the current study will be placed in a broader perspective and limitations and future work will be discussed.

We hope this dissertation will highlight the potential of natural compounds with their beneficial multi-targeting potential as alternatives for the treatment and prevention of breast cancer and provide insight into the advantages of targeting ERβ in combination with ERα for breast cancer treatment and prevention.

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Chapter 2

Literature review

Cancer is the second leading cause of death in developing countries. Amongst women, breast cancer is the most commonly diagnosed malignancy amounting to 23% of all diagnosed cancer cases world-wide (1–5). In general, women from developing countries such as South Africa have been associated with a poorer prognosis in comparison to those from well-developed countries (6). It seems reasonable to postulate that the high cost of modern breast cancer therapies, both for treatment and prevention, have played a large role in this. Therefore, there is a need to better understand the cause of breast cancer development and progression to prevent breast cancer and/or develop breast cancer treatments that are not only highly effective, but highly tolerable, practical and affordable.

For the purpose of this literature review, I will discuss the role of estrogen in breast cancer development and progression and elaborate on the mechanisms involved that are currently targeted for the prevention and treatment of breast cancer, discuss the drawbacks associated with these endocrine therapies and discuss alternative approaches involving natural compounds to overcome these drawbacks. Furthermore, I will discuss the potential of SM6Met, a *Cyclopia subternata* (Honeybush) extract, as alternative to current treatments, which is the overarching topic of this dissertation.

2.1 Breast cancer development and progression with the focus on estrogen

The process whereby a normal cell is transformed into a cancer cell is known as carcinogenesis, characterized by the dysregulation of the balance between the processes of proliferation and cell death (apoptosis). The process of carcinogenesis for multiple types of cancer, including breast cancer, is said to consist of three phases distinguished as: (1) initiation, (2) promotion and (3) progression (Fig. 2.1) (7–9).

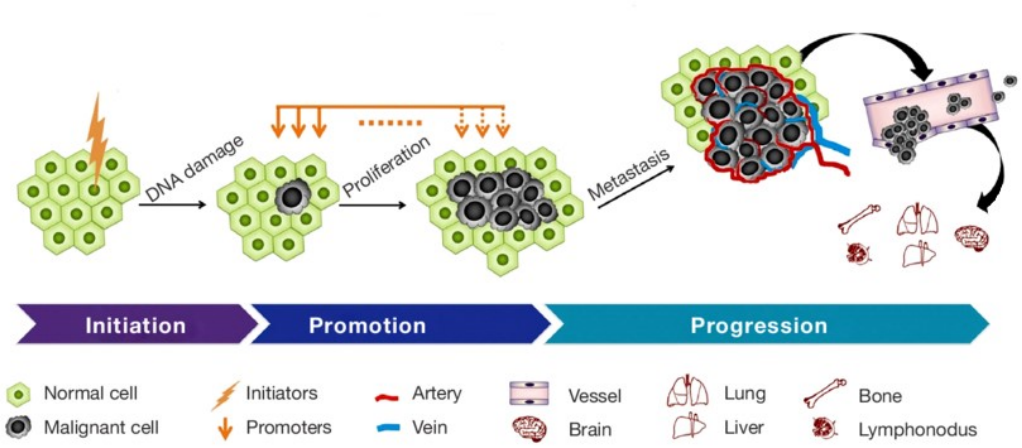

Figure 2.1: Three phases of carcinogenesis. Figure adapted from a study by Liu *et al*. (10).

2.1.1 Initiation

The irreversible process of initiation involves damage to the genomic DNA of a normal cell by various genetic, environmental and lifestyle factors such as UV radiation, tobacco smoke and genotoxins, which has either not been repaired or inadequately repaired, resulting in mutated cells that may undergo further division to form various clones of daughter cells that also carry the mutation and are susceptible to promotion (8, 11, 12). Furthermore, a defective DNA maintenance system that fails to detect DNA damage, repair damaged DNA and inactivate carcinogenic molecules that may inflict DNA damage, may also increase the probability of cancer initiation (13–17).

With regard to breast cancer, there is a strong association between elevated circulating levels of the sex steroid hormone, estrogen, and breast cancer development and progression (18–20). Interestingly, the most widely accepted risk factors for breast cancer i.e. obesity (21, 22), early menarche (23, 24), late age at first full-term pregnancy (23, 25) and late-onset menopause (23, 25, 26) all contribute to excessive and cumulative exposure to estrogen. High levels of estrogen, both endogenous and exogenous, increases cell proliferation and decreases the time apportioned for DNA maintenance, thereby, subsequently increasing the probability of DNA damage and mutations. Moreover, catechol estrogens are metabolites of the endogenous estrogens, estrone (E1) and estradiol (E_2), which can be oxidised to form reactive quinones that have been shown to inflict DNA damage, the basis of carcinogenesis (27–35).

2.1.2 Promotion

The promotion phase is characterized by increased clonal expansion of the already mutated cells. By driving alterations of gene expression the initiated cell acquires certain hallmark characteristics that favour the increase of cell growth (36, 37). These hallmark characteristics include the ability to stimulate own growth, the ability to resist anti-growth signals and the ability to evade programmed cell death (Fig. 2.2) (17, 36, 38).

With regard to breast cancer, mutations in the gene that encodes for aromatase (a key enzyme involved in estrogen synthesis) may lead to overproduction of estrogen, which has been shown to induce breast tumour growth (39–43). Moreover, mutations in the estrogen receptor subtypes, ERα and ERβ, have been shown to promote breast cancer development (44, 45). Estrogen elicits its effects through these nuclear receptor transcription factors (46–49) and their structure and molecular function will further be discussed in section 2.1.5. However, to clarify the implications of ER subtype mutations, it is important to know that previous studies have shown a positive correlation between ERα, mediator and driving component of cell proliferation, and the development of breast cancer (50), while, ERβ has been shown to oppose the proliferative actions of ERα and may act as a tumour suppressor (51, 52). A mutation within the ERα subtype, the Tyr537Asn mutation, has been shown to enable ligand independent activation of ERα, thereby increasing proliferative signalling and contributing to hormone independent tumour growth (53). The gene that encodes for ERα has been shown to be amplified within proliferating breast cancers (54). Researchers hypothesize that the elevated receptor levels create cells that are hypersensitive to growth factors in order to sustain proliferative signalling (17). Furthermore, an ERβ gene mutation that reduces the expression of ERβ has been identified, which, taking into consideration the function of ERβ, may lead to a greater risk of breast cancer development (55).

Numerous other mutations, although not discussed, have been shown to contribute to the promotion of breast cancer by not only contributing to the cell's ability to sustain proliferative signalling (BRCA1 [82], CDK's [38], Myc [38], cyclin D1 (56, 57), but also contributing to the cell's ability to evade growth suppressors and apoptosis (PTEN [38], Akt [38], Bcl-2 [37,38], p35 [37,38]) (17, 58, 59).

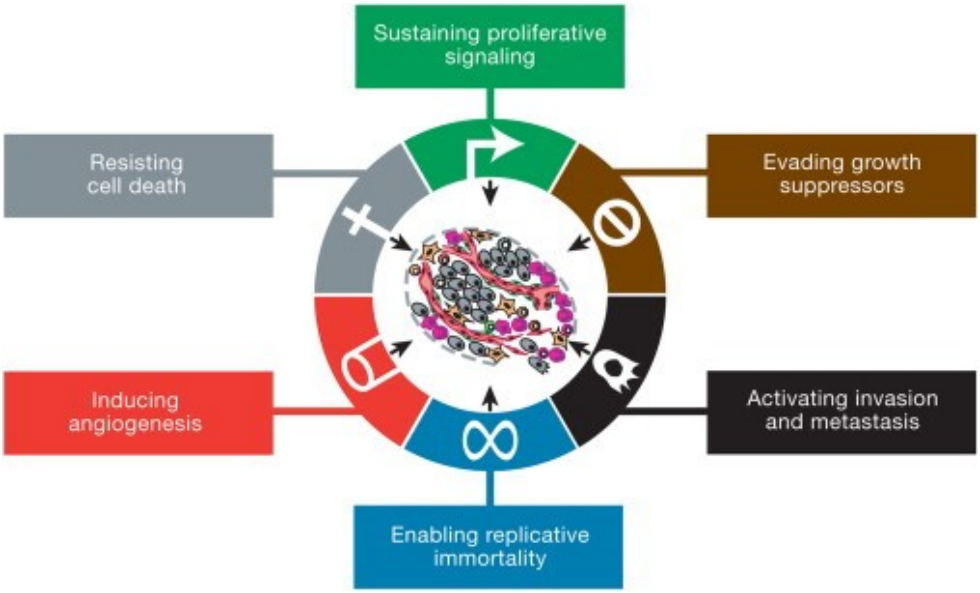

Figure 2.2: The six hallmarks of cancer. Figure taken from a study by Hanahan and Weinberg (16)**.**

2.1.3 Progression

The third and last phase of carcinogenesis, progression, is characterized by an increase in malignant potential through procurement of the remaining hallmark of cancer characteristics i.e. enabling replicative immortality, induction of angiogenesis and the activation of invasion and metastasis (Fig. 2.2) (8, 16).

Normal cells do not have the capability to replicate indefinitely and are limited to a certain number of divisions before becoming non-proliferative (senescence) (17, 60). DNA sequences at the ends of chromosomes known as telomeres shorten with every cell division and activate senescence when they reach a certain length. Senescence is an irreversible process, which is always followed by apoptosis (17, 61). Telomere length is, therefore, involved in the procurement of replicative immortality (17, 62) and cells may bypass senescence through manipulation of enzymes that increase telomere length. For example increased levels of telomerase, the enzyme responsible for adding telomeric repeats to telomeres, has been identified in cancer cells (63–65). Moreover, with regard to breast cancer, estrogen has been found to increase telomerase activity in the MCF-7 human breast cancer cell line via its cognate receptor, ERα, thus, implicating estrogen and its mechanism of action in the progression of breast cancer (66, 67).

To support its immortal replicative ability acquired during cancer progression, the tumour cells kick-start a process called angiogenesis, which involves the formation of new blood vessels to continuously supply the tumour cells with adequate amounts of nutrients and oxygen as well as to remove metabolic waste and carbon dioxide (17, 68–70). Hypoxia, a state of oxygen deficiency, has been shown to play a pivotal role in the induction of angiogenesis (68). With regard to breast cancer, previous studies have shown that estrogen via ERα increases the production of nitric oxide (NO) and consequently hypoxia (68). Furthermore, increased levels of hypoxia-inducible factor 1 (HIF-1), a transcription factor known for its involvement in the initiation of angiogenesis, have been identified in breast cancer (68). Increased expression of ER and HIF-1 has been shown to be interconnected (68), suggesting that estrogen via ERα plays a role in angiogenesis through induction of hypoxia or up-regulation of angiogenesis initiators.

The final hallmark acquired during cancer progression is the ability of primary tumour cells to invade neighbouring tissue and spread (metastasize) to distant parts in the body (17, 71, 72). Metastasis is a complex multistep process that starts with local invasion (invasion of cells into adjacent tissue). Thereafter, the cells invade nearby lymphatic and blood vessels (intravasation), travel through the circulatory and lymphatic systems (transit), exit the system to invade the secondary site (extravasation) and form of a new colony of tumour cells also known as a secondary tumour (micrometastases and colonization) (17, 71, 73). However, if any of the steps are not accomplished the whole process may be inhibited (73). Therefore, all the above mentioned steps are potential therapeutic targets to curb metastasis, the leading cause of death amongst breast cancer patients (74–76).

It is clear that estrogen and its cognate receptor plays a key role throughout the development and progression of breast cancer, making estrogen and the ER obvious therapeutic targets for both prevention and treatment of breast cancer. Before I discuss the treatments, I would like to further elaborate on estrogen synthesis and signalling.

2.1.4 Estrogen synthesis and signalling

Estrogen is most commonly known as the female sex hormone due to its role in the regulation of female reproductive activity as well as the development of secondary female sex characteristics. However, estrogen is also involved in the regulation of many other physiological systems i.e. reproduction, bone integrity, cardiovascular health, behaviour and cognition. Therefore it is not surprising that estrogen is involved in many diseases like cancer, cardiovascular disease and osteoporosis (77). Estrogens have been shown to play a protective role in diseases such as coronary heart disease, Alzheimer's and osteoporosis, while being associated with poor prognosis in prostate, breast, ovarian and endometrial cancers (78).

Estrone (E_1), 17β-estradiol (E_2) and estriol (E_3) are the three major endogenous estrogens that naturally occur in a female's body, however, E_2 has been shown to be about 12 times more potent than E_1 and about 80 times more potent than E_3 (79, 80). In pre-menopausal women, these estrogens are predominantly (±95%) produced in the ovaries in response to follicle stimulating hormone (FSH), while the rest is produced in peripheral tissues like the breast, liver, adrenal gland and adipose tissue from steroid precursors (81–84). In post-menopausal women, however, estrogens are predominantly produced via peripheral conversion of steroid precursor molecules (73). In the classical pathway of estrogen steroidogenesis, androstenedione is synthesized from cholesterol through a series of intermediates via catalysed reactions in the theca cells of the ovaries, where-after androstenedione enters the granulosa cells were it is either converted through aromatase (CYP19A1) into E_1 or via 17βHSD into testosterone, which in turn can be converted to E_2, through the action of aromatase (85–87). Once synthesized and secreted, the estrogens are transported via the blood stream bound to albumin and sex hormone binding globulin (SHBG) to their target tissues (e.g. vagina, bone, liver, breast and uterus (88–91)) were the free estrogen can ultimately elicit its biological effects (92–97).

Although there are several other pathways, estrogen elicits its biological effects predominantly through the classical signalling pathway (Fig. 2.3), which involves E_2 binding to the ER. Upon E_2 binding to the inactive ERs, mostly located in the nucleus (95, 96, 98), the ER will undergo a conformational change resulting in hetero- or homo-dimerization depending on the co-expression of the ER subtypes. This in turn enables the ER-ligand complex to bind to the estrogen response element (ERE) present in the promoters of ER regulated genes leading to the direct or indirect (via co-factor proteins) recruitment of transcription machinery to up- or down-regulate the expression of these target genes (99). It is clear that the ERs are essential for estrogen signalling and the structure and function of the ERs will therefore be discussed further in more detail.

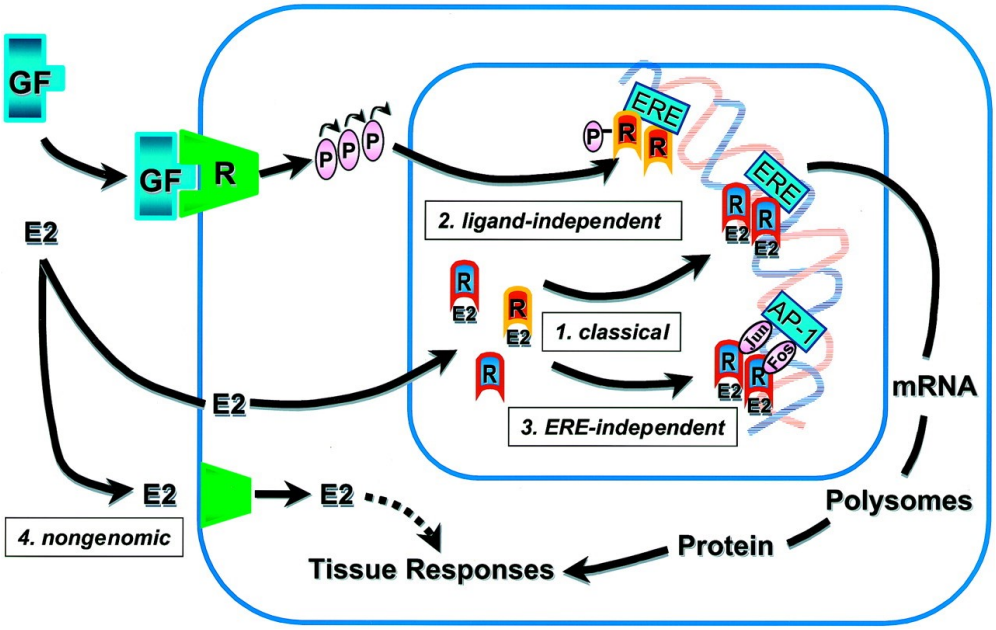

Figure 2.3: The four signalling pathways through which estrogens produce a biological response. The classical pathway involves estrogen binding to its receptor (R), the ER, to form complexes that bind to the ERE which in turn up- or down-regulates estrogen responsive genes (1). The ligand independent pathway involves the activation of intracellular kinase pathways via growth factors (GF) or cyclic adenosine monophosphate (cAMP) which activates the ER through phosphorylation (2). The ERE-independent pathway involves other DNA-bound transcription factors (TFs) like Fos or Jun that tether the activated ER to genes with alternative response elements like activating protein 1 (AP-1) (3). The non-genomic signalling pathway involves the stimulation of intracellular cascades or signalling pathways via activation of putative membrane-associated binding sites (4). Figure taken from Hall et al. (100).

2.1.5 Estrogen receptors

The ERs may be divided into two categories: the nuclear ER, which belongs to the steroid receptor family that forms part of the nuclear receptor family, and the membrane estrogen receptors (mERs). This study will, however, focus on the former. The nuclear receptor family represents the largest family of transcription factors that display structural homology (101–105). Up until the discovery of a second ER subtype (named ERβ) in 1996, it was thought that estrogens elicited their biological effects solely through one ER subtype (renamed ERα) (106, 107).

2.1.5.1 ER subtype structure and homology

The two ER subtypes are encoded by two different genes situated on different chromosomes. In humans, ERα is located on chromosome 6 and is encoded by the gene ESR1 (108), while ERβ is located on chromosome 14 and is encoded by the gene ESR2 (109). Although the human ERβ (hERβ) protein containing 530 amino acids (59.2 kDa) is shorter than the human ERα (hERα) protein containing 595 amino acids (66.2 kDa), the two ER subtypes share a significant degree of sequence homology (Fig. 2.4) especially in the DNA- and ligand-binding domains (110, 111). Both ER subtypes consist of five functional domains named A/B to F from the N-terminus to the C-terminus (Fig. 2.4), with the three major domains

being the C-domain or DNA binding domain (DBD), the E-domain or ligand binding domain (LBD) and the activation domains (AF), A/B and E domains (112).

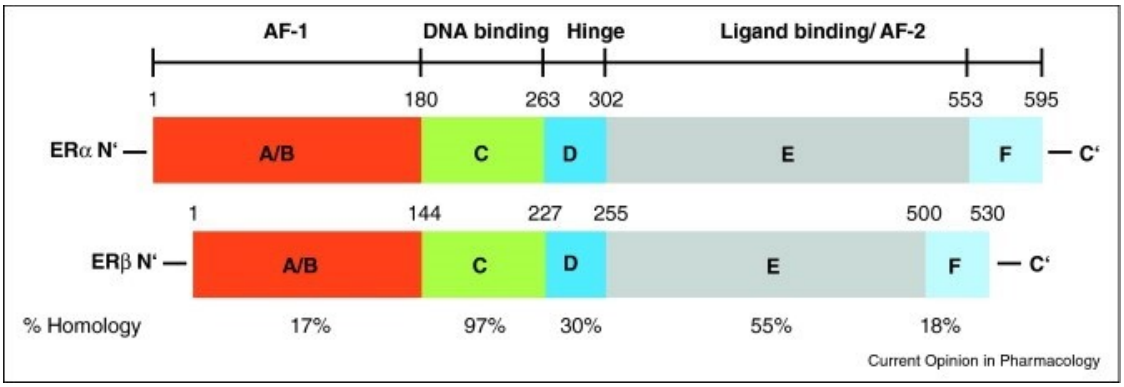

Figure 2.4: Basic domain structure and homology of ER subtypes, ERα and ERβ. Schematic representation of the homology of the functional domains of the human ER subtypes. The DNA binding domain has the highest homology, while the A/B domain containing AF-1 has the lowest homology. The numbers above the structures indicate the amino acid position, while the numbers at the bottom indicate the percentage sequence homology between the subtypes. Figure taken from Leitman *et al*. (113)

The DBD is involved in DNA binding and is highly conserved between the two ER subtypes, therefore, both receptors recognize and bind to the same DNA response elements (EREs). The DBD contains two zinc-finger motifs that entail a motif situated at the C-terminal of the first zinc finger known as the proximal box (P-box) which is involved in DNA interaction and specificity and a second motif situated at the N-terminal of the second zinc finger known as the dimerization box (D-box) which is involved in receptor dimerization (98, 114–116).(116) The LBD is positioned close to the C-terminal end of the receptor and is involved in many processes beyond ligand recognition and binding, for example heat shock protein (HSP) release, dimerization, nuclear localization, co-regulator interaction and activation of transcription [65,127,130]. Because the LBD is less conserved (only 55% homology) than the DBD, E2 has a higher binding affinity towards ERα than ERβ (117), however, this does not apply to all ligands. Phytoestrogens, for instance, generally have a higher binding affinity for ERβ than ERα (118). Furthermore, the transcription activation-function-2 (AF-2) site is located within the LBD, which in association with transcription activation-function-1 (AF-1), located in the N-terminal domain, play important roles in co-activator recruitment, which is present in both ER suptypes (114, 119–121).

2.1.5.2 ER subtype function and distribution

In terms of physiological function, previous studies have characterized ERα, specifically in breast cancer cells, as mediator and driving component of cell proliferation, especially in the presence of estradiol (50) and have shown that more than 50% of all breast cancer cases can be ascribed to over expression of ERα (51, 89). Although, to date, ERβ is not as well characterized as ERα, it has been shown that ERβ has tumour suppressor characteristics in some cancer types, like breast cancer, including anti-proliferative effects (51, 52, 122). Although both ER subtypes are able to stimulate transcription of E_2 target genes

through the classical pathway of binding to the ERE and stimulating co-regulator recruitment, ERα generally elicits a higher degree of transactivation than ERβ (115, 123–125).

Both ER subtypes are widely expressed throughout the human body (Fig. 2.5) and in some tissues the ER subtypes are co-expressed, however, in some cases they are not expressed in the same cells within a particular tissue (126). Furthermore, the period of ER subtype expression may also differ. For example, during development of the uterus and pituitary gland only ERβ is expressed and once the tissue is mature only ERα is expressed (127). ERα is predominantly expressed in the endometrium, vagina, ovarian stromal cells, liver, breast, hypothalamus and pituitary (128), while ERβ is predominantly expressed in ovarian granulosa cells, kidney, prostate, epididymis, heart (129), lung, hypothalamus, and bladder (130). Although, both ER subtypes are found in breast tissue, ERα expression is up-regulated, while ERβ levels are down-regulated during carcinogenesis. Thus, the ERα:ERβ ratio is higher in breast cancer tissue than in normal breast tissue (131, 132). It is clear that the ERs are important in estrogen signalling and play a role in breast cancer development and progression, therefore, in the next section I will discuss breast cancer therapies that target the ERs.

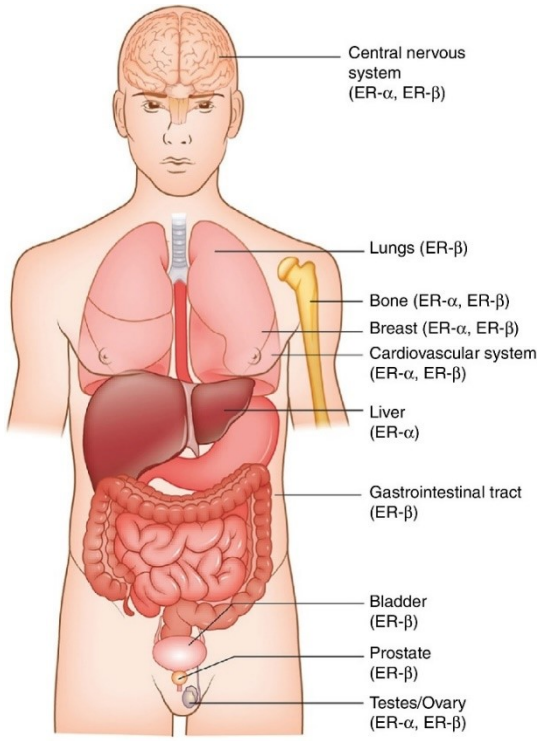

Figure 2.5: Estrogen receptor (ER) tissue distribution in the human body. This simplified diagram was taken from a study by Warner *et al.* (111).

2.2 Estrogen receptor targeted therapy for breast cancer treatment and prevention

Breast cancer tumours that express steroid hormone receptors are known as hormone receptor positive (HR^+) or hormone dependent breast cancer, which accounts for approximately 70% of breast cancers (133). However, the term HR^+ breast cancer usually refers to the expression of the ER (ER-positive [ER^+] breast cancer) as ER is the transcription factor that predominantly drives oncogenesis in HR^+ breast cancer (134). Breast cancer is generally treated with surgery, followed by chemotherapy or radiation, however, ER^+ breast cancers are more often treated with endocrine therapy agents, as neo-adjuvant (prior to surgery) or adjuvant (in addition to surgery) treatment. Current endocrine therapies include selective estrogen modulators (SERMs), selective estrogen receptor down-regulators (SERDs) and aromatase inhibitors (AIs) (135–137). Since this study will focus on hormone therapies that target the ERs and not the synthesis of E_2, aromatase inhibitors, this will not be discussed as it is beyond the scope of this project.

2.2.1 Selective estrogen receptor modulators (SERMs)

SERMs were discovered during scientific studies to develop new contraceptives in the 1950s. It was then discovered that SERMs are structurally similar to endogenous estrogen, which allows them to interact with the ER, leading to the study of the effects of SERMs on hormone dependent breast cancer as a potential treatment option. However, unlike estrogens, SERMs may act like agonists or antagonists depending on the target tissue (124, 138–141), meaning that their effects are tissue specific. Most SERMs bind to the ER subtypes with equal affinity, however, the ER subtypes are not equally distributed throughout the human body and are not functionally equal, thereby influencing the agonist or antagonist properties within different target tissues. Other factors that influence the tissue selectivity of SERMs include cell type, ligand-dependent conformational changes and co-factor recruitment (141–143).

In general, the majority of SERMs elicit protective effects in the bone (ER agonist activity) and breast (ER antagonist activity), while the effects of SERMs on the uterus are known to vary. Due to their protective effects in the bone and breast, SERMs are most commonly used to treat breast cancer and post-menopausal osteoporosis. However, due to the vast array of severe side-effects associated with SERM-treatment, the search for the “ideal SERM” that would elicit strong anti-estrogenic effects to protect against endometrial- and breast cancer, while eliciting estrogenic effects in the bone to protect against osteoporosis (a known side-effect of currently used breast cancer treatments) is ongoing. SERMs may be classified according to their chemical structure as triphenylethylene, benzothiophene, or benzopyran compounds. Additionally the triphenylethylene SERMs, including tamoxifen and its derivatives, are also regarded as first-generation SERMs, while benzothiophene SERMs include compounds that are classified as second-generation SERMs (like raloxifene) or third-generation SERMs, and benzopyran compounds are fourth generation SERMs (144).

Currently there are only two main classes of SERMs approved for clinical use namely the first-generation triphenylethylene derivatives, tamoxifen (145) and toremifene (146, 147), both used for treatment and prevention of breast cancer (148, 149); and the second-generation benzothiopene derivative, raloxifene, used for treatment and prevention of osteoporosis (150) with the added benefit of reducing the risk of breast cancer development in post-menopausal women (151). The first generation SERM, tamoxifen, was discovered in 1967 and still remains the most commonly used hormone therapy for ER^+ breast cancer treatment. Tamoxifen acts as an antagonist of ER in breast tissue thereby reducing breast cancer incidence and development, while acting as an ER agonist in the bone and endometrium (152–154), thereby protecting the bone, yet increasing endometrial cancer risk through increasing endometrial hyperplasia and cell growth. Tamoxifen is also associated with other severe side-effects such as increased risk of strokes, hot flushes and pulmonary embolism (140) that instigated efforts to develop further generations of SERMs. Toremifene (TOR), is structurally similar to tamoxifen (Fig. 2.6), with a single chloride in the side group being the only difference (155). TOR, like tamoxifen, is most commonly used for the treatment of breast cancer, however TORs are generally used for treatment of breast cancer in postmenopausal women as it has been found to interfere with contraception and may cause fetal harm (156). Due to the structural and functional similarities between TOR and tamoxifen, TOR has a similar side-effect profile to tamoxifen, therefore, it is not perceived as an improvement over tamoxifen (157, 158). The second generation SERM, raloxifene, is as effective as tamoxifen in reducing risk of breast cancer development, and although it is also associated with adverse side-effects like hot flushes and blood clots, raloxifene does not increase risk of endometrial cancer (159).

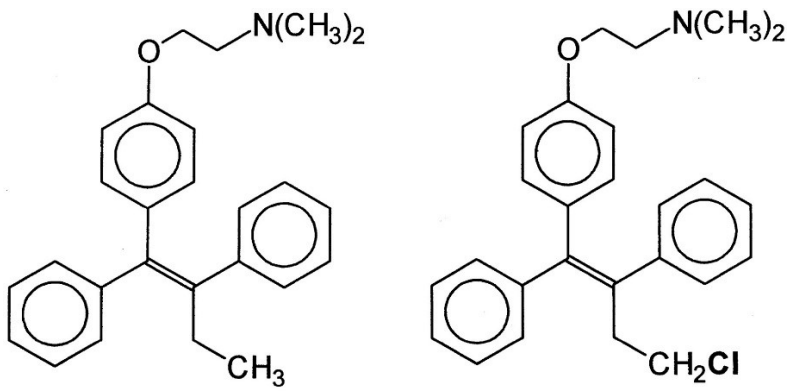

Figure 2.6: Chemical structures of tamoxifen and toremifene. Figure taken from Shibutani *et al*. (10).

Unfortunately, in addition to the adverse side-effects associated with tamoxifen and raloxifene, most of the patients treated with endocrine therapy eventually develop resistance, resulting in disease progression and mortality (160). Statistics show that about 30% of ER^+ tumours show no response to tamoxifen (*de novo* resistance), while the majority of tumours that were initially responsive to tamoxifen treatment, develop resistance (acquired resistance) within two to five years (161).

The unwanted side-effects of SERMs together with the accumulating evidence of the anti-proliferative role of ERβ (52, 122) instigated the investigation and development of ER subtype selective SERMs, like the ERα selective antagonist known as methyl-piperidino-pyrazole (MPP) (162, 163) and the ERβ selective agonists including diarylpropionitrile (DPN), (163, 164), prinaberel (ERB-041) (165, 166), the plant extract MF101 (167), and liquiritigenin (168). However, it is speculated that the ideal subtype selective SERM or rather selective estrogen receptor subtype modulator (SERSM) would possess dual activity, by selectively antagonizing ERα (169), while transcriptionally activating ERβ (164, 167, 168, 170), stabilizing the protein levels of ERβ and down-regulating the protein levels of ERα, as well as demonstrating anti-inflammatory properties by inhibition of pro-inflammatory genes to ultimately decrease the risk of development of post-menopausal osteoporosis (164, 171).

2.2.3 Selective estrogen receptor down regulators (SERDs)

The prospect of down-regulating ER protein levels to attenuate excessive estrogen signalling for therapeutic purposes lead to the development of selective estrogen receptor down-regulators (SERDs). SERDs elicit their function by competitively binding to the ER and inducing a structural change of the ER to a more hydrophobic state, which in turn stimulates proteosomal degradation (172, 173). However, the ideal SERD would function by selectively down-regulating only ERα protein levels, while up-regulating or stabilising ERβ protein levels, to ultimately decrease ERα-dependent breast cancer cell proliferation.

The SERD, fulvestrant (ICI 182,780 or Faslodex) was discovered in the search for a pure ER antagonist and functions by downregulating the protein levels of ERα, while stabilizing the protein levels of ERβ (174). Notably, the majority of ER^+ breast tumours that have become resistant to tamoxifen still remain responsive to treatment with fulvestrant, leading to its approval by the FDA as a second line treatment for tamoxifen resistant ER^+ breast carcinomas (175), thereby indicating that estrogen and the ERs continue to play an important role in regulation of tumour growth even in resistant tumours.

Despite fulvestrant's effectiveness as a second line therapy, its poor systemic exposure, lack of oral bioavailability and association with minor to moderate severity side-effects including, but not limited to, menses disturbances, headaches, nausea, weakness, diarrhoea, hot flushes and vomiting, eliminates fulvestrant as the ideal breast cancer treatment and has led to the development and investigation of newer generation SERDs like etacstil, brilanestrant and elacestrant, which incorporate SERM activity and are known as SERM/SERD hybrids (SSHs). However, none of the SSH's are currently available in the clinic, as elacestrant is currently undergoing clinical trials, while brilanestrant and etacstil were discontinued during clinical trials for non-scientific reasons (176–178).

2.3 Endocrine therapy resistance

Endocrine therapy is perhaps the longest standing, well-tolerated and effective systemic therapy for both adjuvant and metastatic breast cancer treatment (179), with the most commonly used endocrine treatments being AIs, SERMs and SERDs. However, *de novo* and acquired resistance to these treatments poses a substantial clinical dilemma. Current investigations on possible mechanisms and cell signalling of endocrine resistance have brought forth important advances in understanding endocrine resistance. Although the mechanisms associated with resistance are very diverse and any alteration of the components involved in cell signalling pathways could give rise to resistance, the growing understanding of these mechanisms provides the foundation for strategy development to overcome resistance.

Although several mechanism of endocrine resistance have been identified in breast cancer over recent years, this section will provide a brief overview of the principal mechanisms summarized in Fig. 2.7. More detailed discussions of these mechanisms may be found in other literature (180–184).

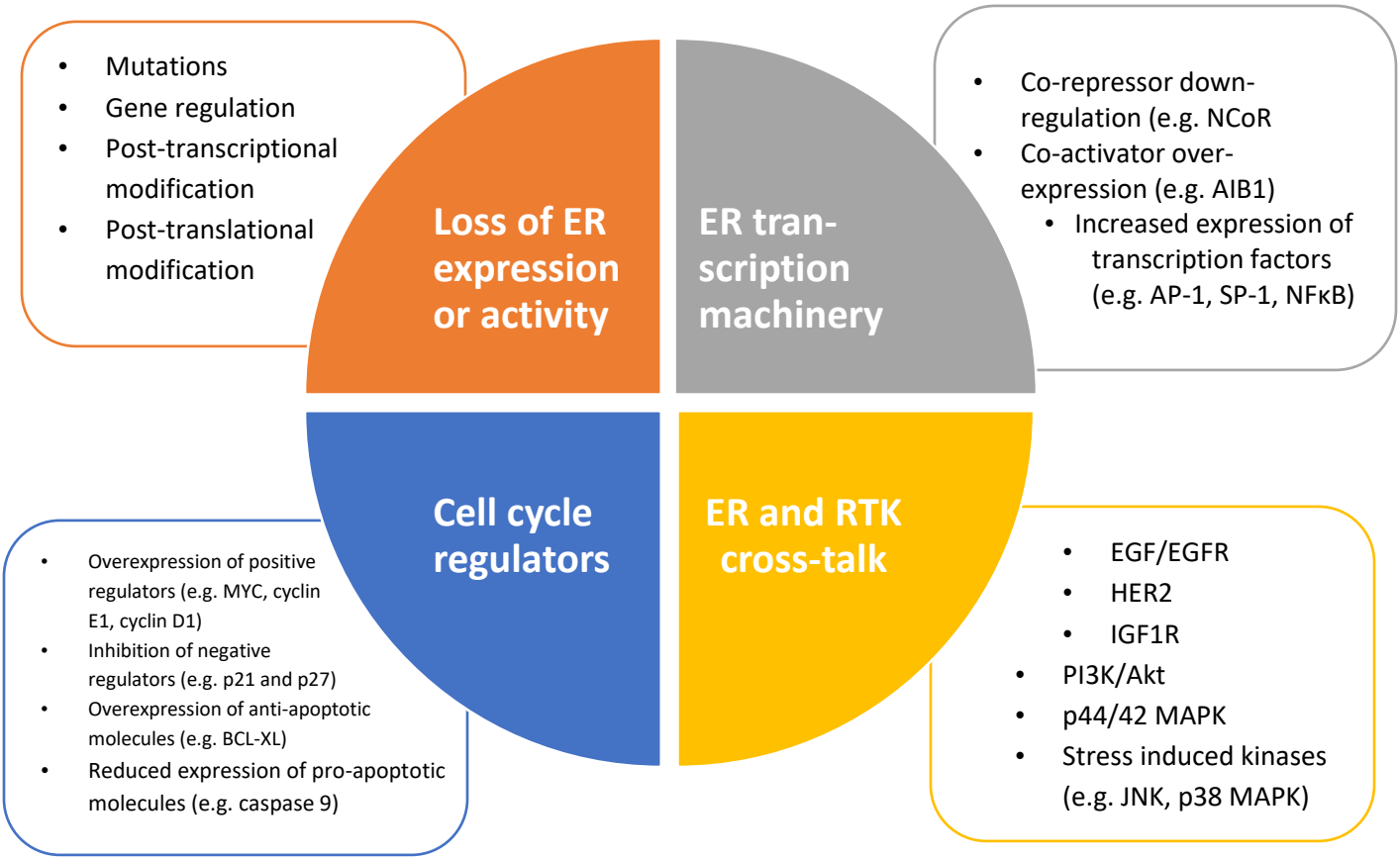

Figure 2.7: Summary of principal mechanisms of endocrine resistance. ER: estrogen receptor; AIB1: amplified in breast 1; NCoR: nuclear receptor corepressor; AP1: activator protein 1; SP-1: specificity protein 1; NFκB: nuclear factor-κB; EGF: epidermal growth factor; EGFR: epidermal growth factor receptor; HER2: human epidermal growth factor receptor 2; IGF1R: insulin growth factor 1 receptor; PI3Ks: phosphatidylinositol 3-kinases; MAPK: mitogen activated protein kinase; JNK: c-Jun N-terminal kinase; BCL-XL: B-cell lymphoma-extra-large; BCL2: B-cell lymphoma 2. Figure drawn by the author, Lorinda van Dyk.

2.3.1 ER expression

To date, ER expression is the leading biomarker for the prediction of endocrine resistance and loss or down-regulation of ER expression (mainly ERα) is thought to be the principal mechanism of innate (*de novo*) resistance, whereas after treatment with endocrine therapy (acquired resistance), loss of ER expression occurs only in 17-28% of resistant breast cancer cases (185–188). Complete loss or down-regulation of ER expression may occur through numerous mechanisms. However, it is thought that ER expression is primarily controlled by ER gene mutations (only 1% of resistant breast cancers) (189–192), post-transcriptional modifications of the ER (e.g. methylation, ubiquitination and phosphorylation) (160, 193–195) and epigenetic modifications of the ER gene or ER associated gene expression that leads to phenotypical changes (196–200).

2.3.2 ER transcriptional components

The ER elicits its biological effects through association with co-regulatory proteins which together form a transcription initiation complex (201). Thus the effectiveness of endocrine therapies could be greatly influenced by changes in this complex of proteins responsible for ER transcriptional activity (202, 203). Firstly, the relative expression levels of co-regulatory proteins is believed to maintain the equilibrium between agonist and antagonist activity of SERMs and changes in the levels of expression of the co-regulators could contribute to endocrine resistance by switching from antagonist to agonist activity and *vice versa*. Results from pre-clinical and clinical studies have suggested that tamoxifen resistance is associated with increased phosphorylation or over-expression of the ER co-activator, AIB1 (otherwise known as NCoA3 or SRC3) (204, 205), whereas experimentally tamoxifen-refractory tumours were found to down-regulate the co-repressor NCoR (206). Secondly, endocrine resistance is also associated with increased activity of transcription factors (TFs) which are important for mediating ER signalling through the non-classical pathway, for example through NFκB, SP-1 and AP-1 (207, 208).

2.3.3 Signal transduction pathway regulation

Cross-talk between different signalling pathways (e.g. cytokine, stress, cell survival (AKT/PI3K) and/or growth factor receptor (GFR) signalling pathways) and the ER signalling pathway have been associated with innate and acquired endocrine resistance. Growth factor receptor tyrosine kinases (RTKs) and their downstream signalling pathways may stimulate cancer growth together with ER signalling or by bypassing the ER signalling pathway. Moreover by modulating ER activity these alternative pathways can directly counter or overcome the inhibitory effects of endocrine therapies.

Over-expression of RTKs such as epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2) and insulin-like growth factor 1 receptor (IGF1R) has been shown to produce tamoxifen resistance in breast cancer cell lines (209–211). Furthermore, clinical evidence has shown that in tamoxifen-treated patients poorer outcomes are associated with EGFR and/or HER2 overexpression (212,

213). In some cases, epigenetic or genetic modification of signal transduction intermediates may cause RTK overexpression (211, 214). Growth factor RTK signalling can be upregulated through the nuclear as well as the non-nuclear activities of the ER, while both the genomic and non-genomic activities of the ER can simultaneously be modulated via numerous RTK pathways. This bidirectional crosstalk has been shown to result in endocrine resistance by activating the ER in the presence of anti-estrogens (215, 216).

2.3.4 Cell cycle and apoptosis regulators

Preclinical and clinical studies have shown a correlation between tumour sensitivity to endocrine therapies and cell cycle regulators (217). More specifically, overexpression of MYC, as well as cyclins D1 and E1, all positive cell cycle regulators, produces endocrine resistance via stimulation of cyclin-dependent kinases important for the G1 phase transition or by inhibiting the stability, activity or expression of negative cell cycle regulators, like p27 and p21, thereby promoting cell cycle progression (218, 219). Furthermore, anti-estrogen mediated apoptosis (cell death) is regulated by apoptotic/survival molecules, thus reduced expression of pro-apoptotic molecules, like caspase 9 and BCL2-interacting killer, or overexpression of anti-apoptotic molecules and proteins, like BCL-XL and Bcl-2, result in deregulation of apoptosis characterized in breast cancer and may also lead to endocrine resistance (220).

2.4 Alternative approaches to overcome the drawbacks associated with endocrine therapy

Earlier I discussed the drawbacks of endocrine therapies that specifically target the ER for breast cancer treatment and prevention, as well as the general mechanisms involved in the occurrence of endocrine resistance in breast cancer. Alternative approaches are, therefore, needed for the treatment of breast cancer to lower side-effects and prevent and/or delay endocrine resistance. The biological rationale behind the multi-targeted approach for breast cancer treatment and prevention is to target tumours by supressing or activating different signalling pathways or processes that are essential for the survival of the tumour, like, for example, inducing apoptosis, inhibiting tumour growth, and inhibiting inflammatory processes, to in turn lower side-effects and delay resistance to the individual drugs (139, 221, 222). In this section I will discuss multi-targeted approaches to breast cancer therapy for the reduction of side-effects associated with SOC endocrine therapies as well as for the prevention or deferment or endocrine resistance. Here I will focus on natural compounds and extracts with multi-targeted mechanisms of action and combination therapies, which include natural compounds.

2.3.1 Natural compounds as therapeutics for breast cancer

For many years natural compounds, otherwise known as phytochemicals, have formed the basis for new anti-cancer drug developments (223). It is evident that many natural compounds elicit anti-breast-cancer activity by acting on multiple signalling pathways (Table 1), thereby providing a multi-targeted approach to breast cancer treatment. Furthermore, natural compounds have been shown to have minimal toxicity and

lower side-effect profiles, *in vitro* and *in vivo*, compared to current synthetic drugs (224). Natural compounds are therefore a promising alternative to current endocrine therapies for breast cancer treatment. In this section, I will briefly highlight some molecular mechanisms whereby natural chemical compounds act against breast cancer.

Epigenetic dysregulation, characterized by phenotypical modification of a cell without modification of DNA sequences, most commonly occurs during initiation and progression of carcinogenesis (225). Epigenetic modification mechanistically involves DNA methylation, acetylation, ubiquitination, phosphorylation and sumoylation of histones and non-coding RNA's or miRNAs, which affect the expression of mammalian genes (226). With regard to breast cancer, epigenetic silencing of tumour suppressor genes and genomic instability during breast carcinogenesis has been linked to abnormal histone modifications and DNA hyper-methylation (227, 228). Source phytochemicals have the ability to modulate epigenetic events and reverse breast cancer causing epigenetic changes. For example, genistein (Table 1) has been found to inhibit DNA methylation and increase tumour suppressor gene expression, specifically up-regulating p21 and p16 expression, in addition to inhibiting DNMT1 expression, the main methyltransferase enzyme responsible for the maintenance of DNA methylation, and re-establishing ERα expression (229, 230). Furthermore, phytochemicals may reactivate tumour suppressor genes and induce cell cycle arrest and apoptosis through epigenetic alterations of specific key transcription factors, kinases and growth factor receptor mediated pathways (231, 232). For example, curcumin (Table 1) has been shown to inhibit class I histone deacetylases (HDACs), histone-modifying enzymes associated with silencing of key genes and transcription factors that regulate physiological functions like cell proliferation and apoptosis, and to upregulate the expression of some miRNAs to reduce the expression of Bcl-2 (233, 234). For more information on the functional roles of epigenetics in breast cancer or more detail on potential epigenetic targets for breast cancer treatment, the reader is referred to the reviews by Lustberg and Ramaswamy (235) and Basse and Arrock (236).

Numerous phytochemicals have been shown to target E_2 signalling through inhibition of aromatase, an enzyme that plays a key role in estrogen synthesis (237, 238). For example, resveratrol (Table 1) has been found to target estrogen signalling by reducing aromatase mRNA expression and by inhibiting transactivation CYP19 promoters (239). The reader is referred to a review by Chumsri *et al*. (240) for further reading on aromatase inhibition.

Furthermore, phytochemicals have shown chemopreventative properties by targeting metabolic processes like the arachidonic acid (AA) pathway, which involves metabolic enzymes implicated in inflammation like lipoxygenases (LOXs), cyclooxygenases (COXs) and phospholipase A2s (PLA2s) (241–243). Specifically, high expression levels of COX-2 have been linked to increased density, invasiveness, metastasis and a poor prognosis in breast cancer (244, 245). Numerous phytochemicals like genistein (Table 1) have been shown to also inhibit the synthesis of metabolic products of the AA pathway like leukotrienes and

prostaglandins (243). For further insight into the AA pathway the reader is referred to a review by Borin *et al*. (246).

Dysregulation of apoptosis, a form of programmed cell death, is a hallmark associated with tumour formation, which also plays a significant role in the development of endocrine resistance (247). Therefore, targeting pathways linked to the evasion of apoptosis are considered to be one of the most significant strategies for cancer treatment, including breast cancer, and to overcome resistance (248). Previous studies have found that phytochemicals, like genistein, epigallocatechin gallate (EGCG), 3,3-diidolylmethane (DIM), curcumin, lycopene, resveratrol, silibin and sulforaphane induce apoptosis in pre-malignant or malignant breast cells through regulation of the two main pathways associated with the activation of apoptosis, i.e. the intrinsic (mitochondrial-mediated) and extrinsic (death receptor-mediated) pathway (Table 1) (248). For example, resveratrol (Table 1) has been found to induce mitochondrial mediated apoptosis by stimulating the p53-dependent pathway and by activating the mitochondrial protein, Smac/DIABLO, thus, promoting caspase activation (caspase-9, and caspase-3) (249–252), while lycopene induces death-receptor mediated apoptosis by increasing the expression of the proapoptotic protein Bax.

The very promising role of natural compounds in treatment and prevention of breast cancer warrants further investigation into potential novel phytochemicals. SM6Met is a sequential methanol extract prepared from the indigenous fynbos plant *Cyclopia subternata* (Fig. 2.8) by sequential extraction using dichloromethane, ethyl acetate, ethanol and methanol and HPLC analysis of the extract indicates the presence of xanthones, flavones, flavanones, dihydrochalcones, benzophenones and phenolic carboxylic acids (51,255). SM6Met has shown great promise for the prevention and treatment of breast cancer. Specifically, previous studies have shown that SM6Met displays phytoestrogenic and anti-mutagenic activity (194), has estrogen receptor subtype selective activity by acting as an ERα antagonist and ERβ agonist, inhibits E_2 induced breast cancer cell proliferation, displays anti-inflammatory behaviour, and antagonizes E_2-induced uterine growth (255). Furthermore, *in vivo* studies have shown that SM6Met acts as chemopreventative agent against LA7-induced and N-MethylN-nitrosourea (MNU)-induced rat mammary gland carcinogenesis (256, 257).

Figure 2.8: *Cyclopia subternata* shoots (left) and the iconic yellow flowers associated with the *Cyclopia* species (right). Photos taken from the South African Honeybush Tea Association (SAHTA) website (258).

Table 1: Summary of multi-targeting molecular mechanisms of natural compounds for treatment and prevention of breast cancer

Natural Chemical Compound or Extract	Major Source	Molecular mechanism of action
Biochanin A	Red clover	• Targets estrogen synthesis by inhibiting aromatase expression (259). • Targets breast cancer cell viability, proliferation and invasion by inhibiting human epidermal growth factor receptor (HER-2) activation, Erk1/2, Akt, mTOR, MMP-9, MT-MMP1 and NF-κB (260).
Curcumin	Turmeric	• Induces apoptosis by increasing the levels of p53, which in turn increases Bax expression leading to an elevated Bax/Bcl-2 ratio (261), • Inhibits breast cancer cell proliferation by downregulating cyclin D, MMP1 and NF-κB transcription (262). • Targets self-renewal in breast stem/progenitor cells through inhibition of the Wnt signaling pathway, a pathway dysregulated in many malignancies, including breast cancer (261). • Has epigenetic activity as it has been shown to inhibit class I histone deacetylases (HDACs) and upregulate the expression of some miRNAs to reduce the expression of Bcl-2, thereby, inhibiting carcinogenesis of breast cancer (233, 234).
3,3-Diidolylmethane (DIM)	Broccoli, cauliflower and cabbage	• Down-regulates genes involved in maintenance of cell growth, cell cycle and apoptosis like survivin, Bcl-2 and cdc25A, while upregulating the cyclin-dependent kinase inhibitor, $p21^{WAF1}$, resulting in DNA damage and cell cycle arrest (263).

		• Targets the arachidonic acid pathway, by specifically inhibiting cyclooxygenase-2 (COX-2, an enzyme known to promote inflammation and carcinogenesis) expression in MCF-7 breast cancer cells (263). • Targets estrogen synthesis by inhibiting aromatase expression (264). • Inhibits angiogenesis, by reducing the accumulation of hypoxia-induced factor (HIF)-1α and subsequently decreases expression of key hypoxia responsive factors, VEGF, furin, enolase-1, glucose transporter-1, and phosphofructokinase in hypoxic tumour cell lines (265).
Emodin	Rhubarb and buckthorn	• Inhibits HER-2/neu-overexpressing breast cancer cell proliferation by inhibiting HER-2/neu tyrosine kinase activity (266).
Epigallocatechin gallate (EGCG)	Green tea	• Induces apoptosis by decreasing aryl hydrocarbon- (AhR-) regulated genes; blocking the ERβ-specific inhibitor, PHTPP; down-regulating the expression of Bcl-2, while increasing Bax levels; increasing production of cytochrome c; increasing Apaf-1 expression; activating caspase-3 and poly(ADP-ribose) polymerase; altering the activity of EGFR; and increasing the expression of proapoptotic genes like p21 and p27, caspase-3, caspase-8, and caspase-9 and TP53 (267–276). • Targets the arachidonic acid pathway, by inhibiting COX-2 expression and activation of NF-κB (277, 278). • Modulates the expression of tumour suppressor genes through epigenetic processes involving DNA methylation and histone modifications, specifically decreases 5-methylcytosine, DNA methyltransferase (DNMT) activity, specifically, DNMT1, DNMT3a, and DNMT3b; decreases histone deacetylase activity; increases levels of acetylated lysine 9 and 14 on histone H3 (H3-Lys 9 and 14) and acetylated lysine 5, 12, and 16 on histone H4; decreases the levels of

		methylated H3-Lys 9; increases the expression of p16INK4a and Cip1/p21; and induces the expression of the epigenetically repressed TIMP-3 gene (275, 276, 279).
Genistein	Soy bean	• Induces apoptosis by up-regulating Bax and p21WAF1 protein; down-regulating caspase-3; blocking the IGF-1R-PI3K/Akt pathway and reducing the Bcl-2/Bax ratio; and enhancing G2/M arrest through activation of the ATM/Chk2/Cdc25C/Cdc2 checkpoint pathway (280–283). • Inhibits cell proliferation and induces cell cycle arrest through up-regulation of ERβ (284). • Targets the arachidonic acid pathway, by inhibiting COX-2 expression, regulating PEG2 by acting as antagonist of AA; inhibiting sPLA2, NF-κB and ERK mediated phosphorylation of p65 and activation of ERα and ERβ (284–288). • Has epigenetic activity as it inhibits DNA methylation and increases tumour suppressor gene expression, specifically up-regulation of p21 and p16 expression, inhibition of DNMT1 expression and re-establishing ERα expression (229, 230).
Lycopene	Tomato, carrot, watermelon, papaya and cherry	• Reduces cell proliferation and induces apoptosis through regulation of growth factor signalling pathways and activation of cell cycle arrest, by specifically inhibiting the phosphorylation of Akt as well as upregulating the proapoptotic Bax without affecting antiapoptotic Bcl-xL; suppressing

		cyclin D1 and upregulating p21; sustaining ERK1/2 activation; increasing p53 expression (289, 290). • Upregulates GSTP1 and demethylates GSTP1 promoter by epigenetic modification, a gene that is silenced in breast cancer by promoter hyper-methylation (291).
Resveratrol	Grapes	• Induces apoptosis, by stimulating the p53-dependent pathway; suppressing the PI3K pathway; regulating Src tyrosine kinase and signal transducer and activator of transcription 3 (STAT-3) phosphorylation pathways; inhibiting Akt phosphorylation, while activating procaspase-9; and activating mitochondrial protein (Smac/DIABLO), caspase-9, and caspase-3 (249–252) • Targets the arachidonic acid pathway, by inhibiting ERβ, COX-2, NQO2, IKK, and GSTP1 (292, 293). • Targets estrogen signalling by reducing aromatase mRNA expression and inhibiting transactivation of I.3 and II CYP19 promoters (239). • Elicits it anti-tumour effects through epigenetic pathways by inhibiting DNMT 3b expression and decreasing RASSF-1αmethylation; activating SIRT1 and acetyl transferase p300; decreasing DNMT1, DNMT3a, DNMT3b, HDAC1, and methyl CpG binding protein 2 (MeCP2) expression (294–297).
Rosmeric acid	Rosemary	• Targets the arachidonic acid pathway, by inhibiting COX-2 expression, blocking AP-1 activation, antagonizing ERK1/2 activation and inhibiting interleukin-8 (IL-8) in the NF-κB pathway (298, 299).
Shikonin	*Lithospermum erythrorhizon*	• Induces apoptosis through multiple pathways like decreasing steroid sulfatase gene expression; activating caspase-3; suppressing NF-κB pathway, Bcl-2, and Bax; down-regulating p65; and inhibiting IκB-α phosphorylation (300, 301).

		• Targets estrogen signalling pathways, by specifically activating ER ubiquitination which in turn activates ER degradation, inhibiting pS2 and c-myc, estrogen responsive gene promoters, and inducing DNA damage by triggering the Nrf2 pathway (302, 303).
Silibin	Milk thistle	• Induces apoptosis through down-regulation of Bcl-2 expression, while up-regulating Atg12-Atg5 formation and increasing beclin-1 expression (304). • Targets the arachidonic acid pathway, by suppressing Wnt/LRP6 signaling; down-regulating TPA-induced MMP-9 expression; and inhibiting COX-2 expression (305, 306).
SM6Met	*Cyclopia subternata* (honeybush tea)	• Inhibits cell proliferation by targeting estrogen signalling, specifically by acting as an ERα antagonist and ERβ agonist (255).
Sulforaphane	Broccoli, water crass, broccoli sprouts, cabbage and kale	• Induces apoptosis through activation of poly(ADP-ribose) polymerase 1 and caspase family proteins; enhancing cyclin B1 expression; inhibition of tubulin polymerization; down-regulation of the NF-κB signaling pathway; reduction of Bcl-2 and phosphorylated Akt serine/threonine kinase expression (307–309). • Targets the arachidonic acid pathway, by inhibiting NF-κB and COX-2 expression, which is mediated by ERK1/2-IKK-α and NAK-IKK- β (310, 311). • Inhibits hTERT (human telomerase reverse transcriptase) through an epigenetic pathway involving DNA methylation and histone modification (309).

2.3.2 Combination therapy approach for breast cancer treatment and prevention

The biological rationale behind combination therapy for breast cancer treatment and prevention is twofold. Firstly, combinations that target the same cellular pathway may result in increased target selectivity and synergistic effects. Synergistic combinations not only increase the efficacy of the therapeutic effect, but also reduce the dose needed to exert the same level of effect (increase in potency), thereby, potentially reducing toxicity (adverse side-effects) and delaying or reducing the risk of resistance development (312). Secondly, combinations of drugs with different molecular targets could delay the process through which cancer cells adapt and subsequently delay the occurrence of resistance (139). However, not all combinations work synergistically or enhance effectiveness, which could lower side-effects or delay endocrine resistance, and thus the development of optimum combinations relies on the understanding and identification of possible mechanisms and cell signalling events involved in the development of endocrine resistance that may be targeted (discussed in section 2.3).

Natural compounds with their beneficial multi-targeting activity (Table 1), have been shown to not only ameliorate side-effects such as nausea, fatigue, anaemia and mucositis arising from currently used chemotherapy or other breast cancer treatments (313), but have also shown synergistic action and/or enhancement of effectiveness when used in combination with other chemotherapeutic or endocrine therapy agents (314). Some examples of combinations with positive or synergistic effects on breast cancer include: the combination of DIM with paclitaxel, a well-known tubulin-targeting chemotherapy agent for breast cancer treatment, that synergistically induces apoptosis (315), curcumin in combination with the PI3K inhibitor, LY290042, that synergistically induces apoptosis (316), and enhancement of chemosensitivity to paclitaxel by the addition of sulforaphane (317). Furthermore, in combination, EGCG increases the bioavailability of doxorubicin (317) or 5-fluorouracil (317), both medications used for breast cancer treatment that targets DNA production, as well as the SOC endocrine therapy agent, tamoxifen (317). Moreover, genistein in combination with doxorubicin displayed synergistic cytotoxic effects (317).

2.5 Conclusion

It is apparent from the literature review that multi-targeted therapies herald a new era for anti-cancer pharmaceuticals as an approach to not only enhance efficacy and subsequently lower side-effects, but to also delay the onset of resistance associated with current endocrine monotherapies. Natural compounds and extracts are of great significance in this field as they have not only been found to elicit their action through a number of pathways without initiating any kind of unusual toxic effect, but also to enhance the effects of conventional chemotherapeutic agents in a synergistic or additive manner. Therefore, more research is needed to optimize the activity of these natural compounds and extracts and to develop them into novel therapeutic agents, either as monotherapies or in combination with other conventional therapeutic agents, for the treatment and prevention of breast cancer.

The distinct physiological functions of the ER subtypes, where ERα promotes breast cancer cell proliferation, while ERβ opposes its activity (52, 122), suggest that a compound or extract with dual SERSM activity that acts as an ERβ agonist and an ERα antagonist would be highly beneficial for breast cancer prevention and treatment. Phytoestrogens are estrogen mimicking compounds derived from plants that have currently gained pharmacological interest as these compounds are found to be ER-subtype selective with a higher affinity for ERβ (318, 319). Visser *et al*. (255) showed that the phytoestrogenic extract, SM6Met, from the native South African plant, *Cyclopia subternata*, displays the above mentioned ideal SERSM properties as well as anti-mutagenic activity, suggesting that SM6Met is a superior candidate to prospectively be developed into a phytoestrogenic nutraceutical for prevention and treatment of estrogen induced breast cancer.

Furthermore, the unknown potential of targeting the ERβ signalling pathway in combination with SOC endocrine therapies that mainly target ERα (137), warrants further investigation into such combination therapies for the prevention and treatment of breast cancer. In addition, such a combination could be beneficial for preventing or delaying resistance as the ER has been shown to still play an important role even after endocrine resistance has occurred (175), SM6Met, containing a wide variety of polyphenols, could modulate the ER via multi-targeted interventions in comparison to conventional anti-breast cancer drugs, such as tamoxifen, and in combination could potentially show synergistic effects and help combat the existing challenges of breast cancer treatment including endocrine resistance and toxicity.

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Chapter 3

SM6Met, a Selective Estrogen Receptor Subtype Modulator (SERSM), synergizes with Tamoxifen, a standard of care treatment (SOC) breast cancer hormone treatment, in reducing breast cancer cell proliferation

3.1 Introduction

Reproduction, bone integrity, cardiovascular health, behaviour and cognition, amongst others, are all examples of physiological systems influenced by estrogens (1). Therefore, it is not surprising that estrogen is involved in various diseases including, amongst others, osteoporosis, cardiovascular disease and various cancer types, including breast cancer (1). The aforementioned physiological systems are regulated by distinct cellular functions like cell growth, gene expression and apoptosis, which are activated in response to estrogen binding to the estrogen receptors (ERs) (2, 3). Estrogen signalling is mediated by estrogen binding to two main intracellular ER subtypes, ERα and ERβ (4, 5). Both ER subtypes are widely expressed throughout the human body. ERα is predominantly expressed in the endometrium, vagina, ovarian stromal cells, liver, breast, hypothalamus and pituitary (6). ERβ, on the other hand, is predominantly expressed in ovarian granulosa cells, kidney, prostate, epididymis, heart, lung, hypothalamus, and bladder (7, 8). Previous studies have characterized ERα, especially in breast cancer cells, as mediator and driving component of cell proliferation in die presence of estradiol (9) and have shown that more than 50% of all breast cancer cases may be ascribed to over expression of ERα (10, 11). In contrast, ERβ has shown anti-proliferative effects on breast cancer cells by opposing the actions of ERα (10, 12). Although to date ERβ is not as well characterized as ERα, it is has been shown that ERβ has tumour suppressor characteristics (13). It is clear that ER signalling is critical in the regulation of differentiation and proliferation of breast cancer cells, therefore, the ER may be targeted therapeutically to ultimately inhibit cancer growth (14–16). ERβ's therapeutic potential and value for breast cancer prevention and treatment is, however, still under investigation and thus current breast cancer therapies target ERα only. Current standard of care (SOC) hormone therapies include selective estrogen modulators (SERMs), selective estrogen receptor down-regulators (SERDs) and aromatase inhibitors (17–19).

SERMs are a group of compounds with the ability to selectively act on the ER as an agonist or antagonist in a tissue selective manner. For example, the first generation SERM, tamoxifen, acts as an antagonist of ER in breast tissue, while acting as an ER agonist in the endometrium (20). The antagonistic effects of tamoxifen in the breast is what makes tamoxifen successful in preventing and treating breast cancer (21–23). The tissue selective agonism/antagonism character of each SERM is influenced by their structure, differential cofactor recruitment, ER subtype tissue expression and ratio, and conformational changes of the ER upon ligand binding (21, 24). Therefore, each SERM has a different tissue selective agonism/antagonism profile, which results in distinctly different biological effects (25). The second generation SERM, raloxifene, for instance, acts as an ER agonist in the bone and liver tissue, while it acts

as an ER antagonist in the breast and uterus (26). Raloxifene is as effective as tamoxifen in reducing the risk of breast cancer development, however it failed as a breast cancer treatment showing no effect in pre-menopausal women and is now most commonly used to treat osteoporosis (27, 28). Despite the advantages of both tamoxifen and raloxifene, they have been associated with adverse side effects like hot flushes and blood clots (29). However, in contrast to tamoxifen, raloxifene does not increase the risk of endometrial cancer (30). In addition to the side-effects caused by current SERMs, some breast cancer cases develop resistance to the SOC therapies like tamoxifen and raloxifene (31–33). Although, the mechanism of resistance is still not completely understood (34, 35), selective estrogen receptor downregulators (SERDs) like fulvestrant, are used as a second line of treatment when resistance occurs (32). SERDs, stimulate proteosomal degradation of the ER upon binding to the ligand binding domain (LBD) of the ER, thereby inhibiting ER signalling and estrogen binding (36, 37). Fulvestrant is a pure anti-estrogen meaning it is an antagonist of both ER subtypes in all estrogen target tissues (38). Although fulvestrant, like tamoxifen, inhibits proliferation of breast cancer, it is associated with a wider range of adverse side-effects such as hot flushes, muscle weakness, vasodilatation, asthenia, headache, back pain, nausea, vomiting, and diarrhoea (23, 30, 39) limiting its use by patients who find the side-effects too severe to continue therapy.

The previously mentioned adverse side-effects related to SERMs (23, 30) and SERDs (39) plus the accumulating evidence of the anti-proliferative role of ERβ (12, 13) has resulted in the search for safer alternative SERMs with the ideal selective ER subtype modulator (SERSM) properties of acting as ERβ agonists and ERα antagonists (30). This has produced a shift in pharmacological interest from tissue selective treatments to developing ER subtype selective ligands for the treatment of breast cancer (14, 40). Resistance has not only triggered interest in the development of novel therapies, but also in the development of combined therapies with current SOC treatments, many of which notably include the use of more natural compounds for the treatment of breast cancer (41). Interestingly, a few studies on combined therapies using tamoxifen as the SOC treatment agent showed synergistic anti-cancer effects (42, 43). Therefore, this chapter will not only focus on validating the ER subtype selectivity of SM6Met, a *Cyclopia subternata* extract, but also investigates SM6Met as a possible alternative to current therapies either as a monotherapy or in combination with tamoxifen.

3.2 Material and Methods

3.2.1 Test panel

17β-Estradiol (E_2), (2)-4-hydroxytamoxifen (the active metabolite of tamoxifen, which will be referred to as 4-OH-Tam in this study) and fulvestrant (Ful) were all obtained from Sigma-Aldrich®, South Africa (Sigma). Methyl-piperidino-pyrazole (MPP) and liquiritigenin (Liq) are products of Tocris bioscience, which were obtained from Whitehead Scientific Pty (Ltd), South Africa. The *C. subternata* extract, SM6Met, was previously prepared by a former laboratory member, J.A.K. Visser (44). E_2, 4-OH-Tam, Ful, MPP and Liq stock solutions were prepared in absolute ethanol (EtOH), while the *C. subternata* methanol extract, SM6Met, stock solutions were prepared in dimethylsulfoxide (DMSO), which was diluted with absolute ethanol to a final concentration of 25%.

3.2.2 Cell culture

The African green monkey kidney fibroblast (COS-1) cells and Human Embryonic Kidney 293 (HEK 293) cells (both from ATCC, United states of America) were maintained in 175cm^2 filter cap culture flasks (SPL Life Sciences) containing, high glucose (4.5g/L) Dulbecco's modified eagle's medium (DMEM) from Sigma, supplemented with 10% (v/v) fetal calf serum (FCS) from Merck, South Africa, 100 IU/ml penicillin and 100 µl/ml streptomycin (1% Penstrep), 44mM sodium-bicarbonate and 1mM sodium-pyruvate (Sigma). The MCF-7BUS human breast cancer cells (45) (a kind gift from A. Soto, Tufts University, Boston, Massachusetts, United States of America) were maintained in 175cm^2 filter cap culture flasks containing, high glucose (4.5 g/L) DMEM, supplemented with 5% (v/v) heat inactivated fetal calf serum (HI-FCS), 1% Penstrep, 44mM sodium-bicarbonate and 1mM sodium-pyruvate.

3.2.3 Plasmids

The expression plasmids for human ERα (pSG5-hERα (46)) and human ERβ (pSG5-hERβ (47)) were kind gifts from Prof. F. Gannon (European Molecular Biology Laboratory, Heidelberg, Germany). The ERE-containing promoter reporter construct (3ERE-pS2-pGL3 (48)) was a kind gift from Dr. B. Belandia (Molecular Endocrinology Laboratory, Imperial Cancer Research Fund, London WC2A 3PX, United Kingdom), while the empty vector plasmid (pGL2-Basic) was obtained from Promega Corporation, Madison, Wisconsin, U.S.A.

3.2.4 Western blot

The HEK 293 and COS-1 cells were plated into sterile 10cm^2 tissue culture dishes (Nest Biotechnology Co., Ltd.) at a density of 2 x 10^6 cells/dish. The cells were incubated for 24hrs to allow them to adhere and settle. On day two, the HEK293 and COS-1 cells were transiently transfected with either 150ng of pSG5-hERα per 10cm plate or 150ng of pSG5-hERβ per 10cm plate using XtremeGENE HPTM transfection reagent from Sigma, in concurrence with the recommended instructions of the manufacturer. In brief, the XtremeGENE HP:DNA (1:3) complex was left to incubate for 30min at room temperature. After the 30min incubation, the transfection complex was added to the cells in a dropwise manner and left to incubate for 24hrs. After the 24hr incubation, the cells were re-plated into 6 well plates (Nest Biotechnology Co., Ltd.) at a cell density of 2 x 10^5 cells/well. On day four, the cells were lysed in 250µl SDS reducing buffer (10% (w/v) SDS, 20% (v/v) glycerol, 0.05% bromophenol blue, 0.2M Tris-HCl (pH 6.8) and 10mM β-mercapto-ethanol) and transferred into 1.5ml microcentrifuge tubes to be boiled for 10min at 96°C before being stored at -20°C. Thawed lysates (15µl) were separated on a 10% SDS-PAGE gel using electrophoresis and transferred to a Hybond-ECL nitrocellulose membrane (Amersham Biosciences, South Africa), which was probed for ERα (1:500), ERβ (1:250) and GAPDH (1:500). After the addition of secondary HRP labelled anti-rabbit antibody for ERβ (1:500), and secondary HRP labelled anti-mouse antibody for ERα (1:200) and GAPDH (1:5000), the proteins were visualized using ECL Western blotting detection reagents (Pierce®, Thermo Fisher Scientific Inc., U.S.A) and the Thermo Scientific™ MyECL Imager. The ERα and the ERβ primary antibodies (ERα (E115), cat# ab32063 and ERβ (EPR3777), cat# ab92306) were purchased from Abcam®, while the GAPDH primary antibody (GAPDH (0411), cat# sc-47724) was purchased from Santa

Cruz Biotechnology, Inc., U.S.A. The secondary anti-rabbit antibody (cat# sc-2005) and the secondary anti-mouse antibody (cat# sc-2030), were both purchased from Santa Cruz Biotechnology, Inc., U.S.A.

3.2.5 Promoter-Reporter Analysis

The HEK 293 and COS-1 cells were plated into sterile 10cm^2 tissue culture dishes at a density of 2 x 10^6 cells/dish. The cells were incubated for 24hrs to allow them to adhere and settle. After the 24hrs, the HEK293 and COS-1 cells were transiently transfected with 1500ng of the ERE-containing promoter reporter construct (3ERE-pS2-pGL3), 4500ng empty vector (pGL2-basic), together with either 150ng pSG5-hERα or 150ng pSG5-hERβ expression vectors using XtremeGENE HP^{TM} transfection reagent, in concurrence with the recommended instructions of the manufacturer as described in section 3.2.4. On the third day, the cells were re-plated into sterile 24 well tissue culture plates (Lasec SA [Pty] Ltd) at a density of 5 x 10^4 cells/well and allowed 24hrs to settle. Thereafter, the cells were induced with increasing concentrations of test compounds dissolved in EtOH or methanol extracts dissolved in DMSO alone (agonist mode) or in the presence of 10^{-11}M E_2 (antagonist mode), all of which were prepared in treatment medium (phenol red free DMEM supplemented with 10% fetal calf serum double stripped with dextran coated charcoal (DS-FCS) and 1% Penstrep so that the final concentration of EtOH did not surpass 0.1% (v/v) and DMSO did not surpass 0.025% (v/v). The cells were induced for 24hrs, before the cells were washed with phosphate buffered saline (PBS) and 50µl of lysis buffer (0.2% (v/v) Triton, 10% (v/v) glycerol, 2.8% (v/v) Tris-phosphate-EDTA, and 1.44mM EDTA) was added to each well. The plates were shaken at 4°C for 15min before being frozen overnight at -20°C. Luciferase activity was determined using the luciferase assay kit from Promega Corporation, Anatech, South Africa in consensus with the manufacturer's instructions. In short, the plates were thawed and placed on ice, after which 5µl of cell lysate from each sample was added to a well in a 96 well white polysterene plate (Sigma, South Africa) and allowed to react with 25µl of luciferase substrate, which was automatically added by the Veritas microplate luminometer (Promega Corporation, Anatech, South Africa). The results were measured in relative light units (RLU's). A further 5µl of cell lysate from each sample was added to a 96-well clear microwell plate and allowed to react with 250µl of Bradford solution for 5min in the dark. Thereafter, the protein content was measured as absorbance values at 620nm. During analysis, the luciferase RLU's were normalized to protein content determined using the Bradford assay (49). Each assay included E_2 as positive control and three negative solvent controls including (1) treatment medium, (2) 0.1% (v/v) EtOH in treatment medium and (3) 0.025% (v/v) DMSO in treatment medium. In agonist mode, the results were expressed as fold induction relative to the average results from the three negative solvent controls as there was no significant difference between them in terms of proliferation potential (Suppl. Figure S1), whereas in antagonist mode, the results were expressed as fold induction relative to the positive control, E_2.

3.2.7 MTT cell proliferation assay

The MCF-7BUS cells were withdrawn from steroids for a week before plating by changing the growth medium to DMEM without phenol red supplemented with 5% fetal calf serum double stripped with dextran coated charcoal and heat inactivated (DS-HI-FCS) and 1% Penstrep. Subsequently, on day one the MCF-7BUS cells were seeded into 96-well tissue culture plates (Nest Biotechnology Co., Ltd.) at a density of 3000 cells/well and allowed 24hrs to settle. The next day the cells were induced with test compounds

dissolved in EtOH or methanol extracts dissolved in DMSO alone (agonist mode) or in the presence of 10^{-11}M E_2 (antagonist mode), all of which were prepared in treatment medium (phenol red free DMEM supplemented with 5% DS-HI-FCS and 1% Penstrep) so that the final concentration of EtOH did not exceed 0.1% (v/v) and DMSO did not exceed 0.025% (v/v). The cells were induced for a period of seven days, wherein there were two retreatments on days three and six. On day eight the colorimetric MTT (3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide from Sigma-Aldrich®) assay was performed as adapted from Verhoog *et al.* (50) and Mfenyana *et al.* (51). In short, after the 7 day incubation period the medium was aspirated and changed to 150µl/well unsupplemented DMEM without phenol red. Thereafter, 50µL of MTT solution at a concentration of 5mg/ml was added to each well and left to incubate for four hours at 37°C. After the four hour incubation step, the medium was removed and 200µL of solubilisation solution (isopropanol) was added to each well. The plate was then covered with foil, shaken at room temperature for 5min, and the absorbance read at 550nm on a BioTek® PowerWave 340 spectrophotometer. Each assay included E_2 as positive control and three negative solvent controls including (1) treatment medium, (2) 0.1% (v/v) EtOH in treatment medium and (3) 0.025% (v/v) DMSO in treatment medium. In agonist mode, the results were expressed as fold induction relative to the average results of the three negative solvent controls as there was no significant difference between them (Suppl. Figure S1), whereas in antagonist mode, the results were expressed as fold induction relative to the positive control, E_2.

3.2.8 Statistical analysis of data

GraphPad Prism® version 5.03 for Windows was used for graphical presentation and statistical analysis. One-way ANOVA with Tukey's multiple comparisons test as post-test was used as statistical analysis method, except for the analysis of two groups (data sets) for which a two tailed unpaired student's t-test was used (as described in all figure legends). Significant statistical difference between groups are indicated with different letters and was calculated as a p-value with levels of significance indicated for each experiment.

3.3 Results

3.3.1 Validation of the ER subtype selectivity of the test panel

To validate the previously demonstrated ERα antagonist and ERβ agonist properties of SM6Met (52), I tested its effects on ERα and ERβ mediated ERE-luciferase promoter reporter activation and compared it to the agonist/antagonist profiles of the rest of the test panel. This includes E_2 , a proven agonist of both ER subtypes with a higher potency toward ERα than ERβ, as shown in both proliferation and ligand binding studies (52–55) as positive control; 4-OH-Tam a proven antagonist of both ER subtypes in breast tissue (12, 56, 57) and fulvestrant a pure antagonist of both ER subtypes in all estrogen target tissues (38, 58, 59) as representatives of current SOC therapies for breast cancer treatment; MPP as representative of a proven ERα selective antagonist (60, 61) and liquiritigenin as representative of a proven ERβ selective agonist (62).

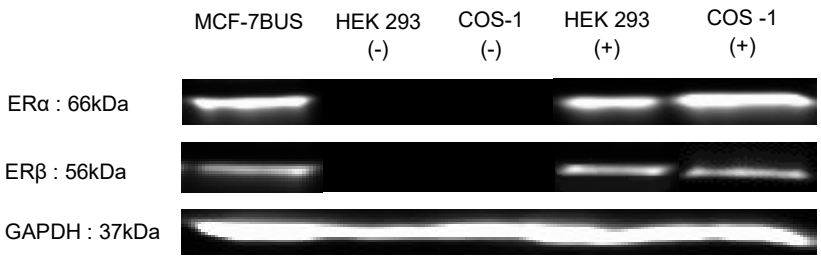

Figure 3.1: Confirmation of successful transfection of the ER subtypes into the HEK293 and COS-1 cell lines. The western blot was performed using antibodies raised against ERα, ERβ and GAPDH (loading control). The MCF-7BUS cell line, loaded into lane 1, is known to endogenously express both ER subtypes and was used as a positive control. Un-transfected HEK293 and COS-1 lysates, loaded in lane two and three, respectively, was used as negative controls and depicted as (-). The transiently transfected HEK293 and COS-1 lysates where loaded in lane four and five, respectively, and depicted as (+).

Experimentally the HEK293 and COS-1 cells, two cell models that do not contain the ER subtypes of interest, were transiently transfected with ERα or ERβ (Figure) together with an ERE-containing promoter reporter construct to evaluate the ERα agonism and antagonism (Figure & Suppl. Figure S2-5) and ERβ agonism and antagonism (Figure & Suppl. Figure S2-5), respectively.

3.3.1.1 The *C. subternata* extract, SM6Met, like the ERβ selective agonist, liquiritigenin, induced transactivation via ERβ

To evaluate ERα and ERβ agonism, the two cell lines, transiently transfected with the respective ER subtypes, were treated with the test panel in a dose dependent manner for 24hrs (Figure, Figure & Suppl. Figure S2-5). E_2 was the only ligand to show agonist activity via both ER subtypes (Figurea and e & Figurea and e). This endogenous ligand not only displayed a significantly ($P<0.05$) 6.3-fold higher potency, but also a significantly ($P<0.001$) 1.6-fold (63.5%) higher efficacy, via ERα than via ERβ in the COS-1 cell line (Table 2 and Table 3). However, in the HEK293 cell line, there was no statistical difference in the potency of E_2 when comparing the two ER subtypes, but a notable ($P<0.001$) 27-fold (2582.6%) higher efficacy through ERβ than via ERα (Table 2 and Table 3).

Out of the two SOC treatments, only 4-OH-Tam showed an effect in agonist mode, albeit an unexpected inverse agonist effect through ERα only in the HEK293 cell line (Suppl. Figure S2B). Although 4-OH-Tam has been shown to be an antagonist of ER in breast tissue (21), no literature was found to corroborate this effect in kidney cell lines or tissue via the ER.

As expected, MPP on its own showed no agonist effect via either ER subtype in either cell line (Suppl. Figure S2D and J and Figure S4D and J). SM6Met, like liquiritigenin, displayed agonist effects via ERβ with no transactivation via ERα (Figurea & e), thereby, validating liquiritigenin as an ERβ selective agonist, whilst confirming that SM6Met acts as an ERβ agonist, albeit significantly less potent than liquiritigenin in both cell lines (Figurec & g and Table 2).

The compounds that displayed ERβ agonist properties may be listed in order of decreasing potency as $E_2 \approx$ liquiritigenin > SM6Met in both cell lines (Table 42) and in order of decreasing efficacy (% induction) as $E_2 >$ liquiritigenin = SM6Met in the HEK293 cell line and $E_2 \approx$ liquiritigenin > SM6Met in the COS-1 cell line (Suppl. Figure S7a & b).

3.3.1.2 The *C. subternata* extract, SM6Met, like the ERα selective antagonist, MPP, inhibited E_2-induced transactivation via ERα

To evaluate ERα and ERβ antagonism, the two cell lines, transiently transfected with the respective ER subtypes, where treated with the test panel in the presence of 10^{-11}M E_2 in a dose dependent manner for 24hrs (Figure, Figure and Suppl. Fig. S2-5). The SOC treatments, fulvestrant and 4-OH-Tam, both acted as antagonists of E_2-induced transactivation of ERα and ERβ in both cell lines (Figureb & f and Figureb & f). Fulvestrant displayed a significantly ($P<0.001$) 327-fold higher potency via ERβ than via ERα in the COS-1 cell line (Figureh and Figureh), with no statistical difference in efficacy. Similarly, in the HEK293 cell line, fulvestrant showed a significantly ($P<0.01$) 15.5-fold higher potency via ERβ than via ERα (Figured, Figured and Table 1 & 2), also with no statistical difference in efficacy. The results showed that 4-OH-Tam elicits its antagonistic effects via both ER subtypes, with no statistical difference in potencies towards ERα and ERβ as well as no statistical difference in efficacies.

Similarly to MPP, SM6Met displayed no antagonistic effects through ERβ (Figureb & f), but displayed significant inhibition of E_2-induced ERE containing-promoter reporter activity via ERα (Figureb & f), thereby validating MPP as a selective ERα antagonist and confirming that SM6Met elicits its antagonistic effects via ERα with approximately the same potency and efficacy as MPP (Suppl. Fig. S6 and Figured & h).

The test compounds that antagonized E_2-induced transactivation via ERα in the HEK293 cell line may be listed in order of decreasing potency as 4-OH-Tam ≈ Ful > SM6Met ≈ MPP (Table 1 and Fig. 3.2d), whereas for the COS-1 cell line may be listed in order of decreasing potency as 4-OH-Tam > Ful ≈ MPP > SM6Met (Table 1 and Fig. 3.2h). The efficacies of the antagonists are not statistically different for either cell line, except in the COS-1 cell line where fulvestrant is significantly different from MPP (Suppl. Figure S6). In summary, when comparing the behaviour of SM6Met with the behaviour of the known agonist and antagonist test compounds, it is clear that SM6Met acts as an ERα antagonist and ERβ agonist, confirming previous work (52).

3.3.1.3 Overall the HEK293 cell line appears to be a more sensitive testing model for ERβ agonism than the COS-1 cell line

In agonist mode, E_2 displayed a significantly ($P<0.01$) 10.8-fold higher potency via ERβ in the HEK293 cell line in comparison to via ERβ in the COS-1 cell line, whereas there was no statistical difference in potency via ERα between the two cell lines. This endogenous ligand was 4.5-fold more effective ($P<0.001$) via ERα in the COS-1 cell line, whereas for ERβ, E_2 was 9.8-fold more effective ($P<0.001$) in the HEK293 cell line. SM6Met and liquiritigenin both displayed higher potencies via ERβ in the HEK293 cell line than via ERβ in the COS-1 cell line, while displaying the opposite in terms of efficacy. In summary, all the compounds that showed agonist effects via ERβ (E_2, liquiritigenin and SM6Met) displayed higher potencies in the HEK293 cell line, while higher efficacies were observed in the COS-1 cell line for liquiritigenin and SM6Met only.

In antagonist mode, fulvestrant displayed a significantly ($P<0.01$) 38-fold higher potency via ERβ in the COS-1 cell line when compared to via ERβ in the HEK293 cells. In addition fulvestrant and MPP displayed a significantly ($P<0.01$) 1.8-fold and 3.3-fold higher potency towards ERα in the COS-1 cell line when compared to ERα in the HEK293 cell line, respectively. Furthermore, 4-OH-Tam displayed a significantly

higher potency via both ERα ($P<0.05$) and ERβ ($P<0.001$) in the COS-1 cell line when compared to the HEK293 cell line. In contrast, SM6Met displayed a significantly ($P<0.05$) 11.3-fold higher potency towards ERα in the HEK293 cell line in comparison to the COS-1 cell line. In summary, when comparing between the two cell lines, no significant difference was found in efficacy via ERα or ERβ for any of the antagonist compounds (fulvestrant, 4-OH-Tam, MPP and SM6Met). With regard to potency, three out of the four ERα antagonists (4-OH-Tam, fulvestrant and MPP) were more potent in COS-1 cells, while both ERβ antagonists (4-OH-Tam and fulvestrant) were more potent in the COS-1 cell line (Figuree and Figuree).

To conclude, we were able to validate and quantify the ER subtype agonist and/or antagonist profiles of the commercially available and well-established test compounds, 4-OH-Tam, fulvestrant, MPP and liquiritigenin, in two cell models, allowing us to confirm and compare the ERα antagonist and ERβ agonist properties of SM6Met. SM6Met proved to be a weaker antagonist of ERα than the SOC therapies (4-OH-Tam and fulvestrant) as well as a weaker ERβ agonist than the commercially available ERβ agonist, liquiritigenin. Interestingly, with regard to the two cell models, all the antagonists for both ER subtypes (except SM6Met) displayed a higher potency in the COS-1 cell line, while all the agonists for both ER subtypes displayed a higher potency in the HEK293 cell line. With regard to the two cell models, the HEK293 cell line proved to be a more sensitive testing model for ERβ agonism than the COS-1 cell line, while the COS-1 cell line proved to be a more sensitive testing model for antagonism of both ER subtypes.

3.3.2 Evaluation of the effect of the test panel on E_2-induced breast cancer proliferation

As described previously ER subtype-selective action could be of great biomedical importance in developing an optimal therapy for breast cancer prevention and/or treatment. Having validated the ER subtype selective properties of SM6Met, I wanted to evaluate the effects of SM6Met not only in a more integrated model where both ER subtypes are co-expressed (Figure), but also on a more physiologically relevant model, namely breast cancer cell proliferation.

3.3.2.1 Fulvestrant and MPP act as inverse agonists in breast cancer cell proliferation

Agonism (Figurea-c and Suppl. Fig. S8) of cell proliferation was determined by using the colorimetric MTT assay after treating the MCF-7BUS cells with the test panel alone in a dose-dependent manner. E_2, as expected, induced cell proliferation in a dose dependent manner with significant induction at a wide range of concentrations from 2.7×10^{-6}µg/ml to 0.27µg/ml (10^{-11}M to 10^{-5}M), with a 2 ± 0.03-fold efficacy and a potency of 7.6×10^{-7} ± 0.19µg/ml (Table 4). In contrast, fulvestrant and MPP both displayed true inverse agonism as they significantly repressed cell proliferation on their own as no exogenous estrogen was present because of the use of phenol red free medium and DS-FCS (Figure S8 C & D). MPP revealed a significantly ($P<0.05$) higher efficacy in inhibiting cell proliferation on its own compared to fulvestrant (Figurea & e and Table 4), however, fulvestrant on its own displayed a much higher ($P<0.001$) potency than MPP (Figurec).

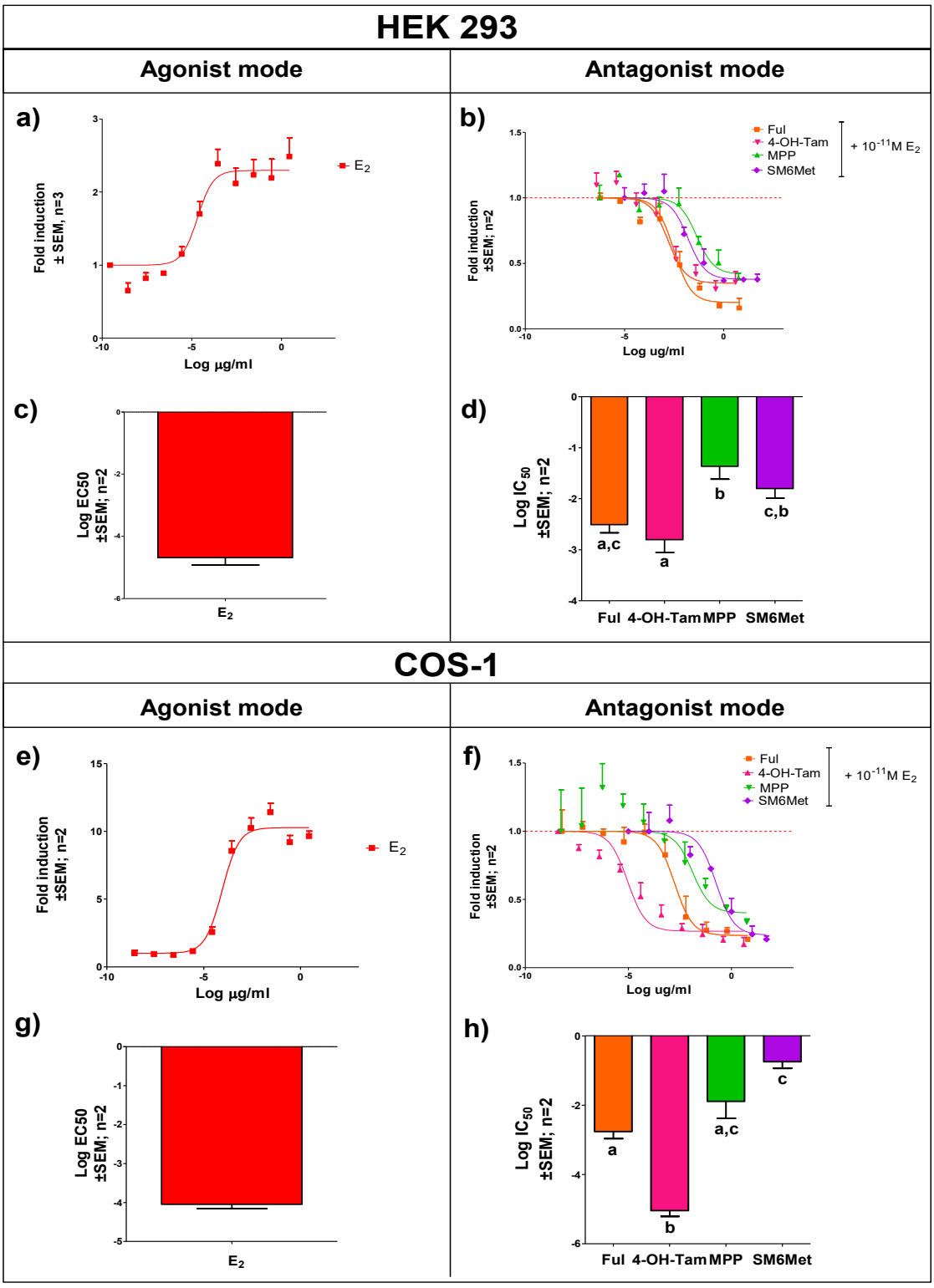

Figure 3.2: Evaluation of the ERα subtype selective agonism and antagonism of the test panel by measuring ERα induced ERE-containing promotor reporter luciferase activity in the HEK293 and COS-1 cells. HEK293 and COS-1 cells were plated into sterile 10cm^2 tissue culture dishes at a density of 2 x 10^6 cells/dish and transiently transfected with pSG5-hERα together with an ERE-containing promoter reporter construct 24hrs after plating. To test agonism (a,c,e & g) the cells were treated with E_2, 4-OH-Tam, fulvestrant, MPP, liquiritigenin and SM6Met in a dose dependant (a & e) manner 24hrs after transfection, whereas antagonism (b,d,f & h) was tested by treating the cells with 4-OH-Tam, fulvestrant, MPP, liquiritigenin and SM6Met in a dose dependant manner (b & f) in the presence of 10^{-11}M E_2 (represented by the red dotted line). Subsequent to the 24hr induction with the test panel, the cells were lysed using passive lysis buffer. Luciferase activity was measured and normalised to protein content, which was determined using a Bradford assay. Statistical analysis of the Log IC_{50} values (d & h) was performed using One-way ANOVA with Tukey's multiple comparisons test as post-tests, where different letters indicate statistical significance ($P<0.05$). The average ± SEM is of two independent biological experiments done in triplicate.

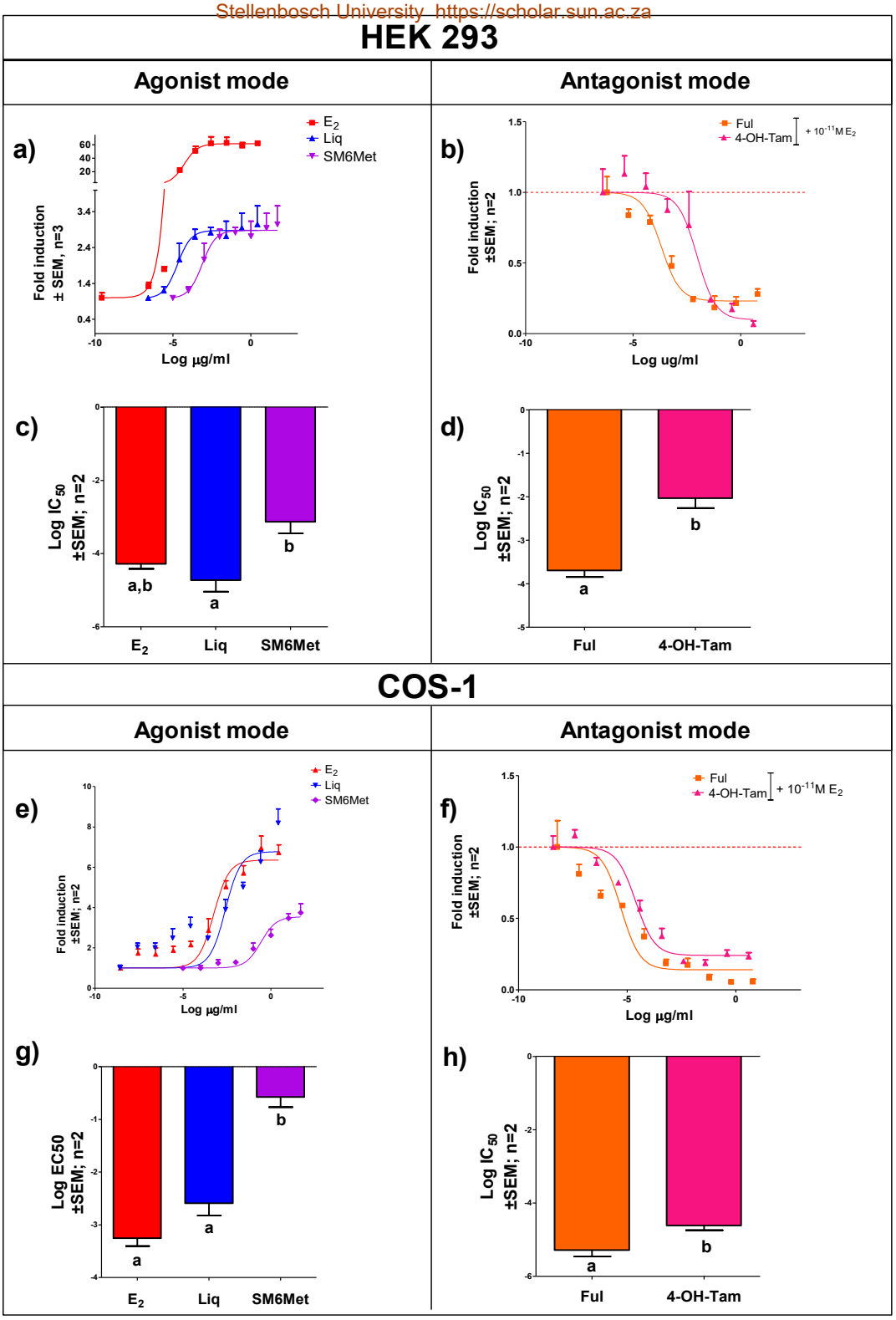

Figure 3.3: Evaluation of the ERβ subtype selective agonism and antagonism of the test panel by measuring the induced ERE-containing promoter reporter luciferase activity in the HEK293 and COS-1 cells. HEK293 and COS-1 cells were plated into sterile 10cm^2 tissue culture dishes at a density of 2 x 10^6 cells/dish and transiently transfected with pSG5-hERβ together with an ERE-containing promoter reporter construct, 24hrs after plating. To test agonism (a,c,e & g) the cells were treated for with E_2, 4-OH-Tam, fulvestrant, MPP, liquiritigenin and SM6Met in a dose dependant (a & e) manner 24hrs after transfection, whereas antagonism (b,d,f & h) was tested by treating the cells with 4-OH-Tam, fulvestrant, MPP, liquiritigenin and SM6Met in a dose dependant manner (b & f) in the presence of 10^{-11}M E_2 (represented by the red dotted line). Subsequent to the 24hr induction with the test panel, the cells were lysed using passive lysis buffer. Luciferase activity was measured and normalised to protein content which was determined using a Bradford assay. Statistical analysis of the Log EC_{50} values (c & g) was performed using One-way ANOVA with Tukey's multiple comparisons test as post-tests, where different letters indicate statistical significance ($P<0.05$). Statistical analysis of the Log IC_{50} values (d & h) was performed using a two tailed unpaired student's test, where different letters indicate statistical significance ($P<0.05$). The average ± SEM is of two independent biological experiments done in triplicate.

Table 2: Summary of ERα agonism and antagonism of the test panel in the HEK 293 and COS-1 cell lines (data from Fig. 3.2 & Suppl. Fig. S2-6)

Compounds	Agonist mode[a]				Antagonist mode[b]				Bio-character[f]
	HEK 293 cell line		COS-1 cell line		HEK 293 cell line		COS-1 cell line		
	Efficacy[c] ± SEM	EC_{50}[g] ± SEM (µg/ml)	Efficacy[c] ± SEM	EC_{50}[g] ± SEM (µg/ml)	Efficacy[d] ± SEM	IC_{50}[h] ± SEM (µg/ml)	Efficacy[d] ± SEM	IC_{50}[h] ± SEM (µg/ml)	
Estradiol (E_2)	100% (2.3 fold) ±0.08	2.1×10^{-5} ±0.24	100% (10.3 fold) ±0.27	8.9×10^{-5} ±0.10	-	-	-	-	Agonist
4-OH-Tam	-16.7% ±0.04	1.8×10^{-4} ±0.24	-	-	65.1% ±0.05	1.6×10^{-3} [e] $ $ ±0.25	73.3% ±0.03	9.1×10^{-6} $ $ ±0.17	Inverse Agonist (HEK 293) Antagonist
Fulvestrant (Ful)	-	-	-	-	79.9% ±0.04	3.1×10^{-3} ±0.16	76.4% ±0.04	1.7×10^{-3} ±0.20	Antagonist
Methyl-piperidino-pyrazole (MPP)	-	-	-	-	58.3% $ ±0.06	4.3×10^{-2} ±0.25	60% ±0.11	1.3×10^{-2} ±0.49	Partial Antagonist (HEK293) Antagonist (COS-1)
Liquiritigenin (Liq)	-	-	-	-	-	-	-	-	No effect
SM6Met	-	-	-	-	62.1% $ ±0.04	1.6×10^{-2} $ $ ±0.19	76.2% ±0.05	1.8×10^{-1} $ $ ±0.19	Partial Antagonist (HEK293) Antagonist (COS-1)

(-) = No effect.

[a]Tested in the absence of E_2.

[b]Tested in the presence of 10^{-11} M E_2.

[c]Efficacy in agonist mode is shown as % activation of ERα mediated ERE-promoter reporter activity, relative to solvent, with E_2 set as 100%. Fold induction of E_2 indicated in brackets.

[d]Efficacy in antagonist mode is shown as % inhibition of E_2-induced ERα mediated ERE-promoter reporter activation.

[e]All antagonists were compared to the full antagonist, fulvestrant ($, represents $P < 0.05$ and $ $, represents $P < 0.01$).

[f]The defining characteristic of the test compound as indicated by the results of the promoter reporter assay. Indicated as partial if the efficacy is significantly less than the full antagonist, fulvestrant.

[g]Concentration of test compound that produces half-maximal ERE-promoter reporter activity.

[h]Concentration of an inhibitor were the ERE-promoter reporter activation of an agonist is reduced to half.

Table 3: Summary of ERβ agonism and antagonism of test panel in the HEK 293 and COS-1 cell lines (data from Fig. 3.3 & Suppl. Fig. S2-5 & S7)

Compounds	Agonist mode[a]				Antagonist mode[b]				Bio-character[g]
	HEK 293 cell line		COS-1 cell line		HEK 293 cell line		COS-1 cell line		
	Efficacy[c] ± SEM	EC_{50}[h] ± SEM (µg/ml)	Efficacy[c] ± SEM	EC_{50}[h] ± SEM (µg/ml)	Efficacy[d] ± SEM	IC_{50}[i] ± SEM (µg/ml)	Efficacy[d] ± SEM	IC_{50}[i] ± SEM (µg/ml)	
Estradiol (E_2)	100% (61.7 fold) ±2.3	5.2×10^{-5} ±0.13	100% (6.3 fold) ±0.27	5.6×10^{-4} ±0.15	-	-	-	-	Agonist
4OH-Tam	-	-	-	-	90.2% ±0.08	9.3×10^{-3} $ $ f ±0.23	75.9% $ ±0.03	2.4×10^{-5} $ ±0.13	Antagonist (HEK293) Partial Antagonist (COS-1)
Fulvestrant (Ful)	-	-	-	-	76.9% ±0.03	2.0×10^{-4} ±0.15	86% ±0.04	5.2×10^{-6} ±0.18	Antagonist
Methyl-piperidino-pyrazole (MPP)	-	-	-	-	-	-	-	-	No effect
Liquiritigenin (Liq)	4.7% *** e ±0.14	1.9×10^{-5} ±0.31	106.6% ±0.49	2.6×10^{-3} ±0.23	-	-	-	-	Agonist (COS-1) Partial Agonist (HEK293)
SM6Met	4.7% *** ±0.13	7.4×10^{-4} * ±0.28	55.8% *** ±0.18	2.7×10^{-1} *** ±0.19	-	-	-	-	Partial Agonist

(-) = No effect.

[a]Tested in the absence of E_2.

[b]Tested in the presence of 10^{-11} M E_2.

[c]Efficacy in agonist mode is shown as % activation of ERα mediated ERE-promoter reporter activity, relative to solvent, with E_2 set as 100%. Fold induction of E_2 indicated in brackets.

[d]Efficacy in antagonist mode is shown as % inhibition of E_2-induced ERα mediated ERE-promoter reporter activation.

[e]All agonists were compared to the full agonist, E_2 (* represents $P < 0.05$ and *** represents $P < 0.001$).

[f]All antagonists were compared to the full antagonist, fulvestrant ($ represents $P < 0.05$ and $ $ represents $P < 0.01$).

[g]The defining characteristic of the test compound as indicated by the results of the promoter reporter assay. Indicated as partial if the efficacy is significantly less than the full agonist, E_2 or the full antagonist, fulvestrant.

[h]Concentration of test compound that produces half-maximal ERE-promoter reporter activity.

[i]Concentration of an inhibitor were the ERE-promoter reporter activation of an agonist is reduced to half.

Interestingly, these inverse agonists inhibited cell proliferation with a similar, not statistically different, efficacy when they were tested in the presence of E_2 (antagonist mode). Fulvestrant was, however, 2.1 times more potent on its own than in the presence of E_2, while MPP proved to be a significantly ($P<0.001$) 11.9 times more potent in the presence of E_2 than on its own. In summary, fulvestrant proved to be more potent in reducing breast cancer cell proliferation than MPP, in both agonist and antagonist mode.

3.3.2.2 The *C. subternata* extract, SM6Met, weakly antagonized E_2-induced breast cancer cell proliferation

Antagonism (Figureb) of E_2-induced cell proliferation was determined by treating the MCF-7BUS cells with the test panel in a dose-dependent manner in the presence of 10^{-11}M E_2. The entire test panel, except liquiritigenin, was able to significantly inhibit E_2-induced cell proliferation (Figureb) with MPP being the most effective (90% ± 0.02 inhibition) and SM6Met being the least effective (19% ± 0.01 inhibition). In comparison to MPP (the most effective antagonist), inhibition of cell proliferation by fulvestrant was a significantly ($P<0.05$) 11% lower than that of MPP, whereas inhibition by 4-OH-Tam was even lower than fulvestrant by a significant ($P<0.001$) 16%. Although, MPP proved to be the most effective antagonist, it also displayed the lowest potency, whereas the extract with the lowest efficacy, SM6Met, proved to be a significantly ($P<0.01$) 11.7 times more potent than MPP. Fulvestrant proved to be the most potent inhibitor of E_2-induced breast cancer cell proliferation since it displayed a potency 28 times ($P<0.01$) higher than 4-OH-Tam, 209 times ($P<0.001$) higher than SM6Met, and 2455 times ($P<0.001$) higher than MPP. In short, the compounds and extract that antagonized E_2-induced cell proliferation may be ranked in order of decreasing potency as fulvestrant > 4-OH-Tam > SM6Met > MPP or in order of decreasing efficacy as MPP > Ful > 4-OH-Tam > SM6Met.

3.3.2.3 Proof of concept that a combination of an ERα antagonist and ERβ agonist is more effective than an ERα antagonist or ERβ agonist on its own in preventing breast cancer cell proliferation.

SM6Met is a selective estrogen receptor subtype modulator (SERSM) in that it selectively antagonizes ERα (Figureb & f), while selectively activating ERβ (Figurea & e), attributes thought to be advantageous in preventing breast cancer cell proliferation (14, 63–67). However, the limitations of natural extracts like SM6Met include, amongst others, lower activity in comparison to purified or synthetic compounds as demonstrated by the low efficacy and potency by which SM6Met inhibited E_2 induced ERE-containing promoter reporter activity (Figure) and breast cancer proliferation (Figure). Therefore, we combined MPP (an ERα selective antagonist) with liquiritigenin (an ERβ selective agonist) in order to mimic the ER subtype selective characteristics of SM6Met. Thus, in a proof of concept study to demonstrate the advantages of a combined therapy consisting of an ERα antagonist and ERβ agonist in inhibiting breast cancer cell proliferation, a constant concentration of MPP (10^{-6}M) was combined with increasing concentrations of liquiritigenin (10^{-11}M to 10^{-7}M), in the presence of 10^{-11}M E_2 (Figure). The IC_{50} concentration of MPP (2.7 x 10^{-1}µg/ml or 10^{-6}M) as determined from the dose response curves in Figure S8 displayed a significant ($P<0.001$) 72% inhibition of E_2-induced cell proliferation, while liquiritigenin with a ±40% average inhibition of E_2-induced cell proliferation at all the concentrations tested, showed no statistically significant increase in inhibition when the concentration of liquiritigenin was increased. However, when the 10^{-6}M of MPP was combined with liquiritigenin, the percentage inhibition of E_2-induced breast cancer cell proliferation

increased in relation to the increase in concentration of liquiritigenin, with significant inhibition compared to 10^{-6}M MPP alone at combinations with 10^{-8}M and 10^{-7}M liquiritigenin ($P<0.05$ and $P<0.001$, respectively).

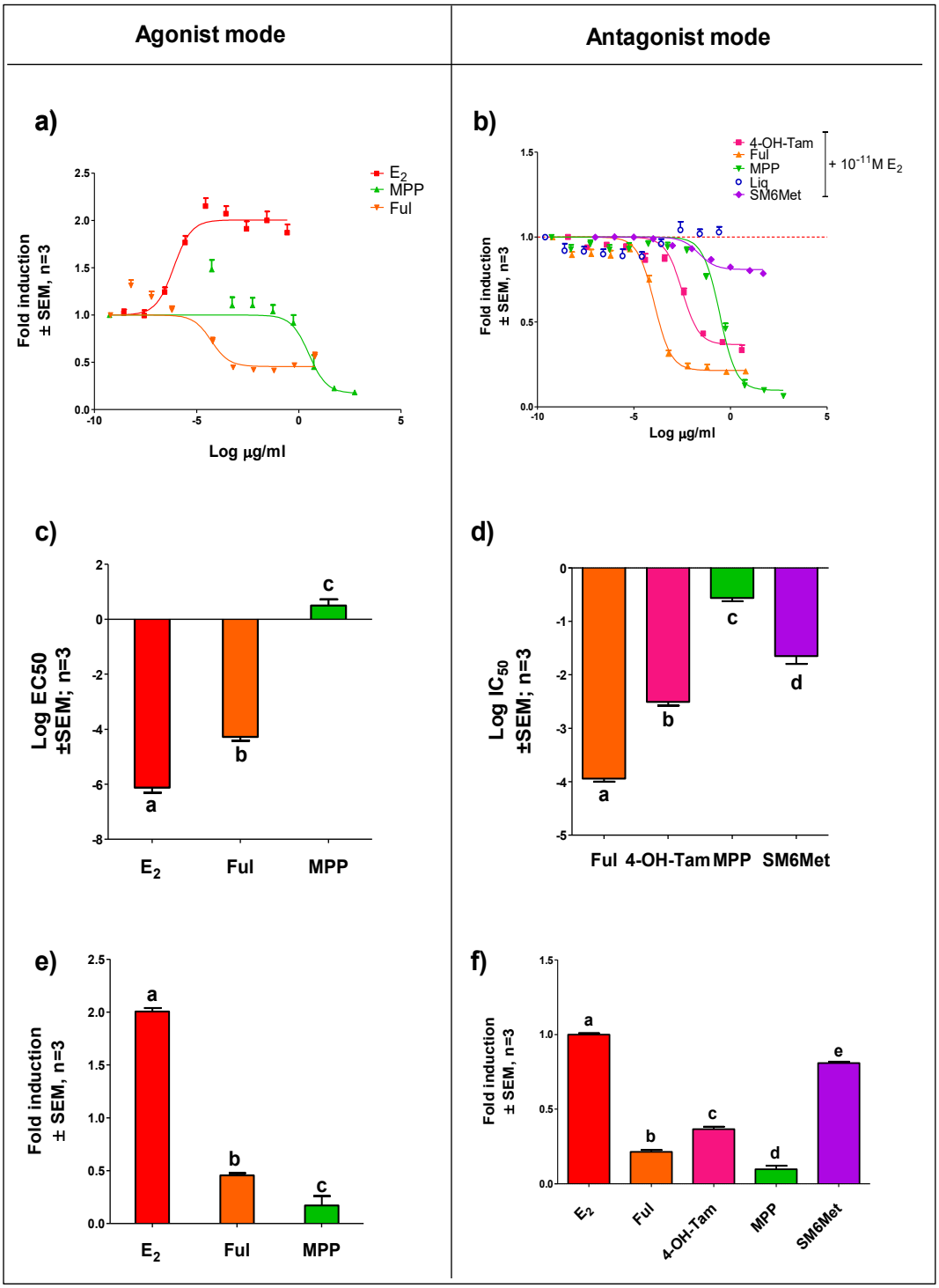

Figure 3.4: Evaluation of the effects of the test panel on E_2-induced breast cancer cell proliferation by means of dose response curves in human MCF-7BUS breast cancer cells, a cell line which endogenously expresses ERα and ERβ. The MCF-7BUS cell were withdrawn from steroids for a week before plating, by changing the growth medium to DMEM without phenol red supplemented with 5% DS-HI-FCS and 1% Penstrep. Thereafter, MCF-7BUS cells were treated with a 4-OH-Tam, fulvestrant, MPP, liquiritigenin and SM6Met in the absence (a, c & e) - agonist mode) and in the presence of 10^{-11}M E_2 (b, d & e - antagonist mode) in a dose dependent manner (a & b) for a period of seven days, wherein there were two retreatments. Thereafter, MTT solution was added to the cells and after a 4hr incubation the medium was removed and the formazan crystals that formed through metabolism were dissolved in isopropanol. Statistical analysis was performed using One-way ANOVA with Tukey's multiple comparisons test as post-tests, where different letters indicate statistical significance ($P<0.05$). The average ±SEM is of three independent biological experiments done in triplicate. E_2 value is represented by the red dotted line (b).

Table 4: Summary of efficacy and potency of proliferation of the test panel evaluated in agonist and antagonist mode in the human MCF-7BUS breast cancer cell line (data from Figure)

Compounds	Agonist mode[a]		Antagonist mode[b]		Bio-character[i]
	Efficacy[c] ± SEM	EC_{50}[j] ± SEM (µg/ml)	Efficacy[d] ± SEM	IC_{50}[k] ± SEM (µg/ml)	
Estradiol (E_2)	100% (2.0 fold) ±0.03	7.6×10^{-7} (±0.19)	-	-	Full Agonist
4OH-Tamoxifen (Tam)	-	-	63% ±0.02	3.1×10^{-3} $ $ $ ±0.07	Partial Antagonist
Fulvestrant (Ful)	-77% ±0.02	5.2×10^{-5} ** [e] ±0.05	79% ±0.01	1.1×10^{-4} ±0.06	Inverse Agonist Full Antagonist
Methyl-piperidino-pyrazole (MPP)	-92% ±0.09	3.2 *** [e] $ $ $ [f] ±0.22	90% ±0.02	2.7×10^{-1} $ $ $ ♦♦ [g] ±0.06	Inverse Agonist Full Antagonist
Liquiritigenin (Liq)	-	-	-	-	No dose response
SM6Met	-	-	19% ±0.01	2.3×10^{-2} $ $ $ ♦♦ ## [h] ±0.15	Weak Antagonist

(-) = No effect.
[a]Tested in the absence of E_2.
[b]Tested in the presence of 10^{-11} M E_2.
[c]Efficacy in agonist mode is shown as % proliferation, relative to solvent, with E_2 set as 100%. Fold induction of E_2 indicated in brackets.
[d]Efficacy in antagonist mode is shown as % inhibition of E_2-induced proliferation.
[e]Statistically different from E_2 (* represents $P < 0.05$, ** represents $P < 0.01$ and *** represents $P < 0.001$).
[f]Statistically different from fulvestrant ($, represents $P < 0.05$, $$ represents $P < 0.01$ and $$$ represents $P < 0.001$).
[g]Statistically different from 4-OH-Tam (♦, represents $P < 0.05$, ♦♦ represents $P < 0.01$ and ♦♦♦ represents $P < 0.001$).
[h]Statistically different from MPP (#, represents $P < 0.05$, ## represents $P < 0.01$ and ### represents $P < 0.001$).
[i]The defining characteristic of the test compound as indicated by the results of the proliferation assay.
[j]Concentration of test panel that produces half-maximal proliferation.
[k]Concentration of test panel were the proliferative effect of the agonist, 10^{-11}M E_2, is reduced to half.

The most effective combination (10^{-7}M liquiritigenin together with 10^{-6}M MPP) displayed 96.5% inhibition of E_2-induced breast cancer cell proliferation, which is 24.5% higher than that obtained with 10^{-6}M MPP on its own. In addition, it is also 6.5% higher than the efficacy of MPP, 33.5% higher than the efficacy of the SERM, 4-OH-Tam, and 17.5% higher than the SERD, fulvestrant, as determined from the dose response curves (summarized in Table 4). In conclusion, it is clear that a mixture displaying both ERα antagonist and ERβ agonist properties is not only more effective at reducing E_2-induced breast cancer cell proliferation than ER subtype selective compounds on their own, but also more effective than some SERMs and SERDs on their own. Therefore, extracts like SM6Met that contain compounds that selectively modulate the ER subtypes show promise for the development of anti-cancer agents.

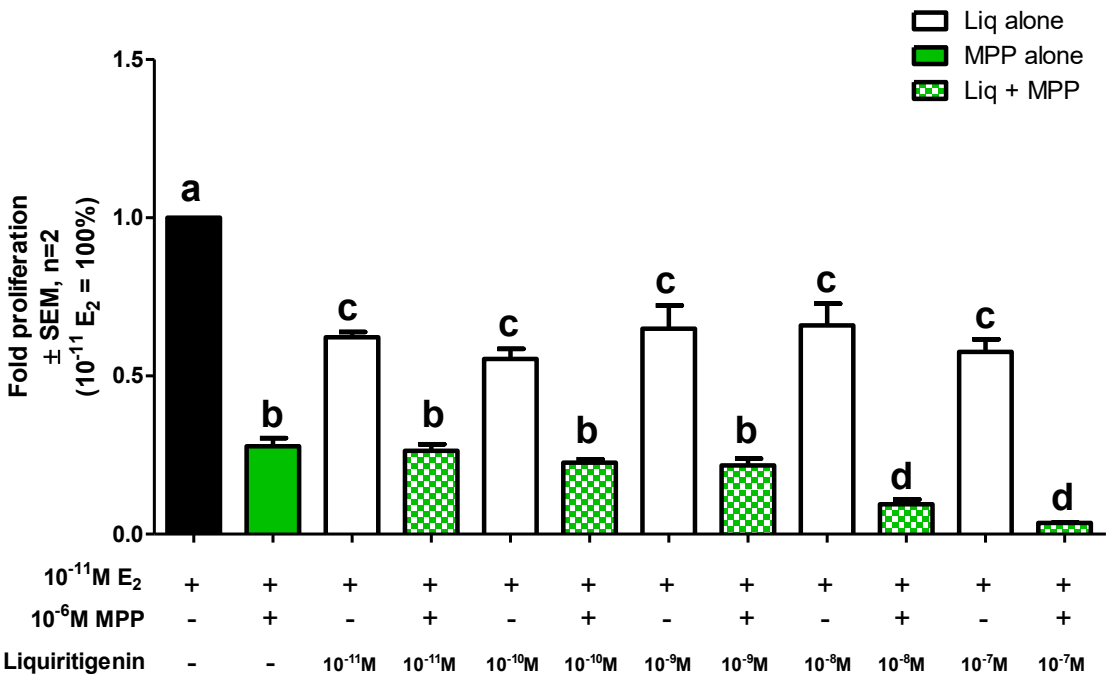

Figure 3.5: Checkerboard analysis of the effect of MPP (ERα antagonist) combined with liquiritigenin (ERβ agonist) on breast cancer cell proliferation. MCF7BUS cells were seeded into 96 well plates at 3000 cells/well on day one, then treated with increasing concentrations of liquiritigenin combined with a constant concentration of MPP, in the presence of 10^{-11}M E_2, on day three and retreated on day six. Thereafter, MTT solution was added to the cells and incubated for a 4hr period. The formazan crystals formed through metabolism indicates the number of viable cells. Statistical analysis was performed using One-way ANOVA analysis of variance with Tukey's multiple comparisons test as post-test, where different letters indicate statistical significance ($P<0.05$). Average ± SEM is of two independent biological experiments done in quadruplicate.

3.3.3 Evaluation of SM6Met and 4-OH-Tamoxifen as combined therapy to inhibit breast cancer cell proliferation

Drug combination studies to achieve enhanced effects is not only popular in cancer research, but also in antibiotic, anti-microbial, immune disease (*i.e.* AIDS) and viral infection studies (68, 69). Combination studies aim to reduce the amount of the drugs needed to elicit the desired response, thereby, possibly reducing the adverse effects (41). This multi-drug concept is used in cancer therapy to target tumours by suppressing or activating different signalling pathways or processes that are essential for the survival of the tumour, like, for example, inducing apoptosis, inhibiting tumour growth, and inhibiting inflammatory processes, and thus in turn delaying resistance to the individual drugs (70–72). To validate improved effectiveness of a drug combination is relatively simple with the checkerboard assay (Fig. 3.6) as the most commonly used method, however enhanced effectiveness does not necessarily mean that the drug combination is synergistic (73). In contrast to the checkerboard assay that only measures enhanced or reduced efficacy, methods to determine synergism measure the degree of enhancement or reduction by the change in potency, also referred to as the interaction index (γ), which will be described later on.

3.3.3.1 SM6Met in combination with 4-OH-Tam displayed significantly higher inhibition of E_2-induced breast cancer cell proliferation than each compound on their own

Investigating enhanced effectiveness of combining SM6Met with 4-OH-Tam at inhibiting E_2-induced breast cancer cell proliferation was the first step in determining the possible synergistic properties of SM6Met.

This was done by treating the MCF-7BUS cells with SM6Met and 4-OH-Tam in a checkerboard fashion in the presence of 10^{-11}M E_2 as depicted below in Fig. 3.6.

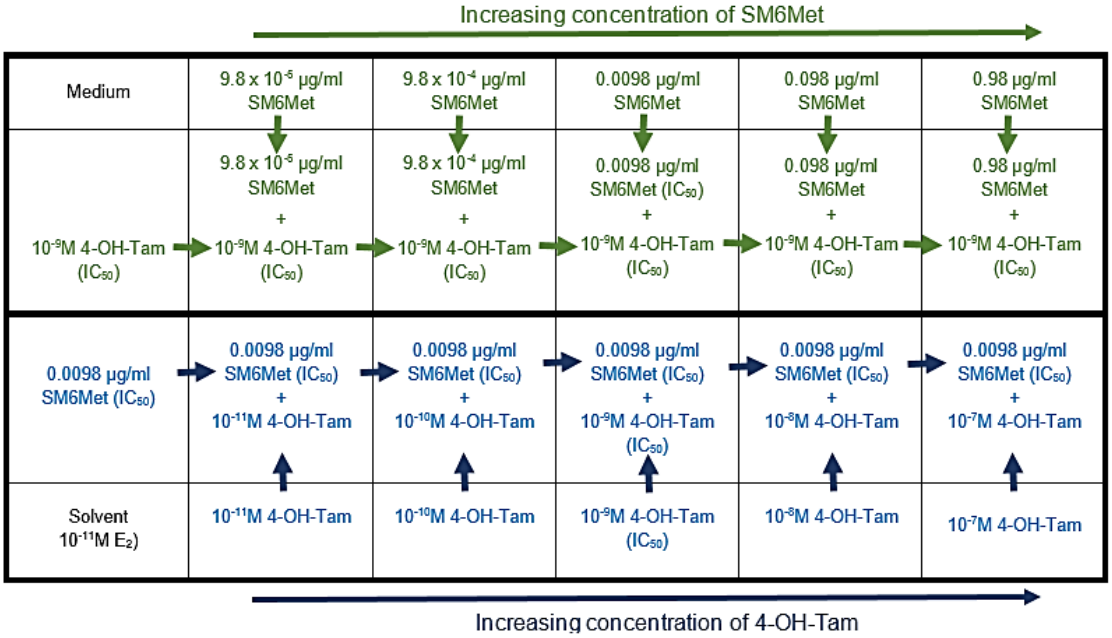

Figure 3.6: Diagram depicting the experimental layout for the treatment step of the checkerboard assay, thereby showing how 4-OH-Tam and SM6Met were combined. Increasing concentrations of SM6Met along the horizontal in row one combined with the constant concentration of 4-OH-Tam in row two and increasing concentrations of 4-OH-Tam along the horizontal in row four combined with the constant concentration of SM6Met in row three. Each block represents 4 wells of a 96 well tissue culture plate.

In FigureA, 10^{-9}M 4-OH-Tam displayed a 44% ($P<0.001$) inhibition of E_2-induced cell proliferation, while SM6Met could only inhibit (22%, $P<0.05$) E_2-induced cell proliferation at the highest concentration used (0.98µg/ml) for this assay. However, when 4-OH-Tam was combined with SM6Met, the % inhibition of E_2-induced cell proliferation increased in relation to the increase in concentration of SM6Met, with significant inhibition compared to 10^{-9}M 4-OH-Tam alone at combinations with 0.0098µg/ml, 0.098µg/ml and 0.98µg/ml SM6Met. The most effective combination (0.98µg/ml SM6Met together with 10^{-9}M 4-OH-Tam) displayed 77% inhibition of E_2-induced cell proliferation, which is 33% higher than the efficacy of 10^{-9}M 4-OH-Tam alone (44% inhibition) and 55% higher than the efficacy of 0.98µg/ml SM6Met alone (22% inhibition). In FigureB, a constant concentration of SM6Met (0.0098µg/ml) was combined with increasing concentrations of 4-OH-Tam. SM6Met at 0.0098µg/ml could not significantly inhibit E_2-induced cell proliferation on its own, while 4-OH-Tam significantly reduced E_2-induced cell proliferation in a dose dependent manner with significant inhibition at concentrations of 10^{-9}M (44%, $P<0.001$), 10^{-8}M (50%, $P<0.001$) and 10^{-7}M (50%, $P<0.001$). However, when the 0.0098µg/ml SM6Met was combined with 4-OH-Tam, the % inhibition of E_2-induced cell proliferation increased in relation to the increase in concentration of 4-OH-Tam. The most effective combination (10^{-8}M 4-OH-Tam together with 0.0098µg/ml SM6Met) displayed 73% inhibition of E_2-induced cell proliferation, which is 23% higher than the efficacy of 10^{-8}M 4-OH-Tam alone (50% inhibition) and 64% higher than the efficacy of 0.0098µg/ml SM6Met alone (9% inhibition). In conclusion, SM6Met in combination with 4-OH-Tam shows promise as a combinatorial therapy that may be used to lower the side-effects or curb resistance, as lower dosages are needed to elicit the same effect as either 4-OH-Tam or SM6Met alone (monotherapy).

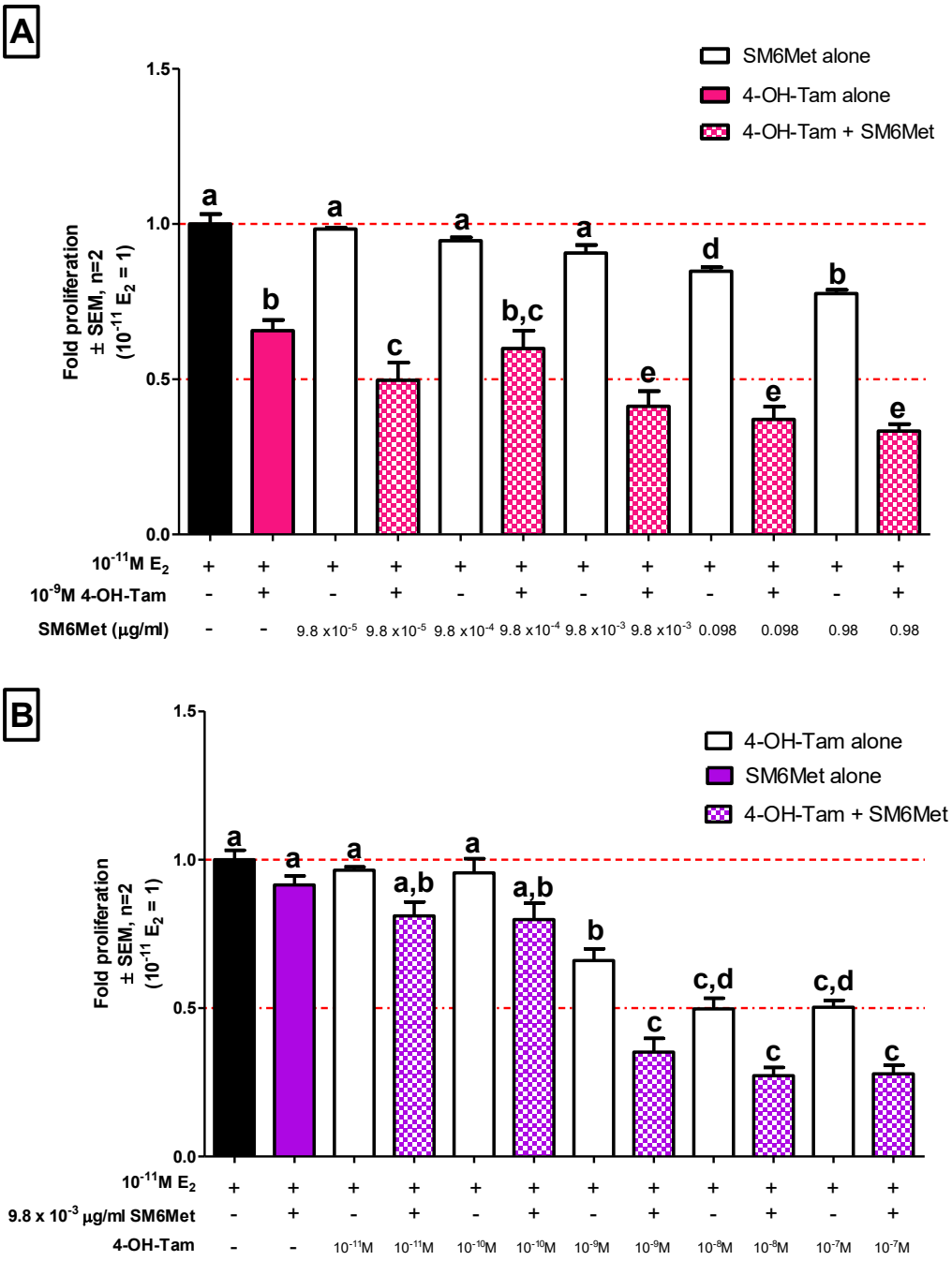

Figure 3.7: Checkerboard analysis of the effect of 4-OH-Tam (a SOC therapy) combined with SM6Met (a SERSM) on breast cancer cell proliferation. (A) MCF7BUS cells were induced with increasing concentrations of SM6Met combined with a constant concentration of 4-OH-Tam (IC_{50} concentration as determined in Figure S8) in the presence of 10^{-11}M E_2 on day three and retreated on day six. On day seven the cells were incubated for 4hrs in MTT solution in order for the live cells to metabolize and form formazan crystals, where the number of crystals formed indicates the number of viable cells. Thereafter, the crystals were dissolved in isopropanol and the absorbance quantified. Statistical analysis was performed using one-way ANOVA analysis of variance with Tukey's multiple comparisons test as post-test, where different letters indicate statistical significance ($P<0.05$). Average ± SEM is of two independent biological experiments done in quadruplicate. (B) MCF7BUS cells were treated with increasing concentrations of 4-OH-Tam combined with a constant concentration of SM6Met (IC_{50} concentration as determined in Figure S8) in the presence of 10^{-11}M E_2 on day three and retreated on day six. On day seven the cells were incubated for 4hrs in MTT solution in order for the live cells to metabolize and form formazan crystals, where the number of crystals formed indicates the number of viable cells. Thereafter, the crystals were dissolved in isopropanol and the absorbance quantified. Statistical analysis was performed using One-way ANOVA with Tukey's multiple comparisons test as post-test, where different letters indicate statistical significance ($P<0.05$). Average ± SEM is of two independent biological experiments done in quadruplicate.

3.3.3.2 SM6Met and 4-OH-Tam, administered together in certain fixed ratio combinations were synergistic in reducing E_2-induced breast cancer cell proliferation

Having shown that the combination of SM6Met with 4-OH-Tam enhances the reduction of E_2-induced breast cancer cell proliferation in comparison to the individual treatments with SM6Met and 4-OH-Tam alone, the interaction index as described by Tallarida *et al.* (74, 75) was used in order to determine whether this combination is synergistic, additive or antagonistic. In short, the interaction index (γ) is obtained via the fixed ratio isobolar method in which the effect level (50%, 75% or 90% inhibition of breast cancer cell proliferation) is determined for each individual drug (monotherapy) and for each combination ratio using dose response curves.

3.3.3.2.1 In combination, SM6Met increases the potency and efficacy of 4-OH-Tam to reduce E_2-induced breast cancer cell proliferation

The analysis of synergism starts with the determination of the potency of each drug (SM6Met and 4-OH-Tam) in the presence of 10^{-11}M E_2 from dose-response curves (Figure). However, to save time we used fixed ratio combination mixtures previously made up by another student in our lab who showed potency values of 4-OH-Tam (3.128 x 10^{-7} µg/ml) and SM6Met (8.841 x 10^{-3} µg/ml) in the presence of 10^{-11}M E_2, similar to mine. These mixtures were created using the IC_{50} concentration of SM6Met in relation to the IC_{50} concentration of 4-OH-Tam and diluted serially as depicted in Figure (2-fold dilution with several concentration points above and below the IC_{50} value of each drug).

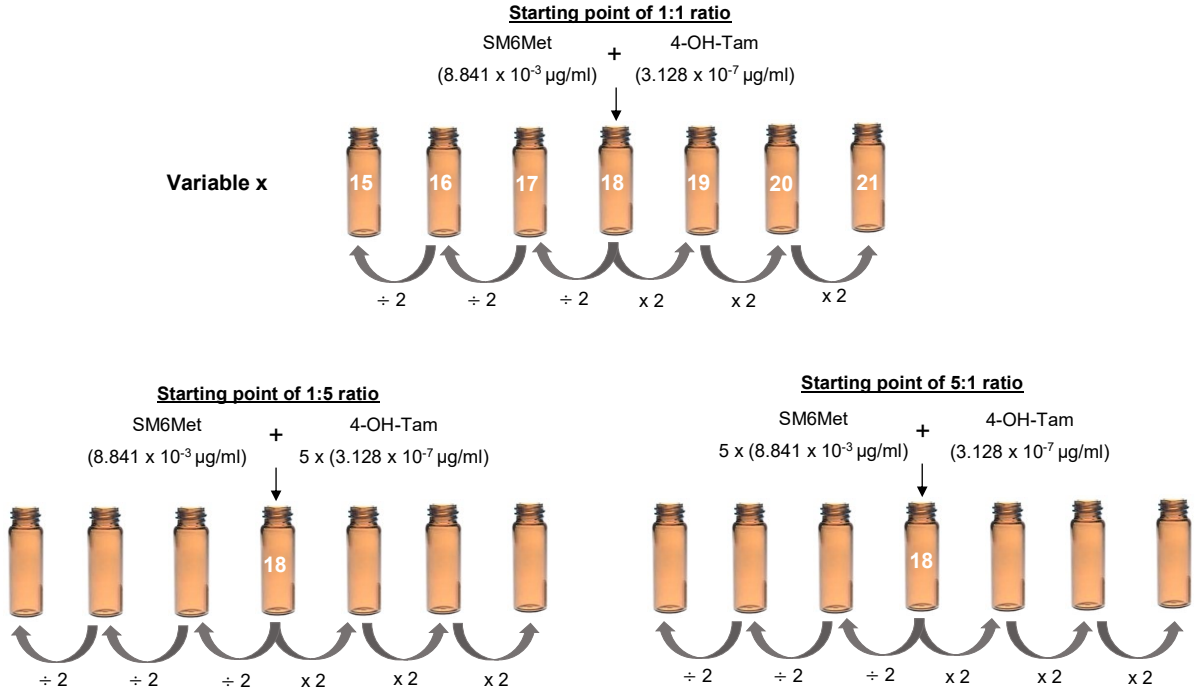

Figure 3.8: Diagram depicting the principle behind the fixed ratio mixtures made by another student in the lab using their IC_{50} concentrations of 4-OH-Tam (3.128 x 10^{-7} µg/ml) and SM6Met (8.841 x 10^{-3} µg/ml) determined using proliferation dose response assays. These concentrations serve as the starting point after which 2-fold serial dilutions of the mixtures were made and used to treat the MCF-7BUS cell line in order to create dose response curves required for the analysis of synergism.

The chosen fixed ratios included a 1:1, 1:5, 5:1, 1:10, 10:1, 1:20, 20:1, 1:50 and 50:1 ratio and the MCF-7BUS cells were treated with a 2-fold dilution series of each ratio, all in the presence of 10^{-11}M E_2. Each dilution was assigned a number (variable x) in order to keep track of the concentrations of each compound and/or extract (SM6Met and 4-OH-Tam) in each vial, subsequently the concentration of each compound can be calculated at any point of the dose-response curve of any fixed ratio combination.

The resulting dose-response curves in FigureA of 4-OH-Tam shift to the left, in the presence of increasing SM6Met concentrations, indicating an increase in potency. The shift seems to be proportional to the amount of SM6Met added, as increasing the amount of SM6Met in the ratio resulted in an increase in the shift and an increase in potency (FigureC), with the exception of the ratio combination of 50:1. Interestingly, in FigureB the curve of SM6Met shifts to the right (decreasing potency) when combined with 4-OH-Tam at a ratio of 1:1 and 1:5. However, when 4-OH-Tam is increased more than five times in relation to SM6Met (1:10, 1:20 and 1:50), the curves shift back in the direction of the monotherapy curve of SM6Met.

There was no statistical difference between the efficacy of 4-OH-Tam alone and the efficacy of 4-OH-Tam in a 1:1 ratio combination with SM6Met (FigureA). However, the combinations with higher SM6Met ratios in relation to 4-OH-Tam, like the 5:1, 20:1 and 50:1 ratios, displayed significantly ($P<0.001$) higher efficacies than the 1:1 ratio combination. As there was no significant difference between the efficacies of the 1:5, 1:10, 1:20 and 1:50 ratios of SM6Met:4-OH-Tam it suggests that increasing the concentration of SM6Met in the combination with 4-OH-Tam, will only improve the efficacy to a certain extent. On the other hand, the addition of 4-OH-Tam to SM6Met in a 1:1 ratio significantly increased the efficacy ($P<0.001$) in comparison to the efficacy of SM6Met alone (FigureB). The combinations with higher 4-OH-Tam ratios in relation to SM6Met were also significantly more efficacious than the efficacy of SM6Met alone. Increase in efficacy seems to be directly correlated to the increase in concentration of 4-OH-Tam in relation to SM6Met, with significant increases in efficacy for the combination ratios of 1:20 and 1:50, but not 1:5 and 1:10, in comparison to the efficacy of the 1:1 combination ratio (FigureB).

In summary, adding SM6Met in combination with 4-OH-Tam enhanced the potency whereby 4-OH-Tam inhibited breast cancer cell proliferation, thereby indicating a possible synergistic effect. The 20:1 combination ratio is considered the best combination ratio of SM6Met:4-OH-Tam, as it had the highest potency and only one combination ratio with a very low potency (1:50) had a statistically ($P<0.05$) higher efficacy.

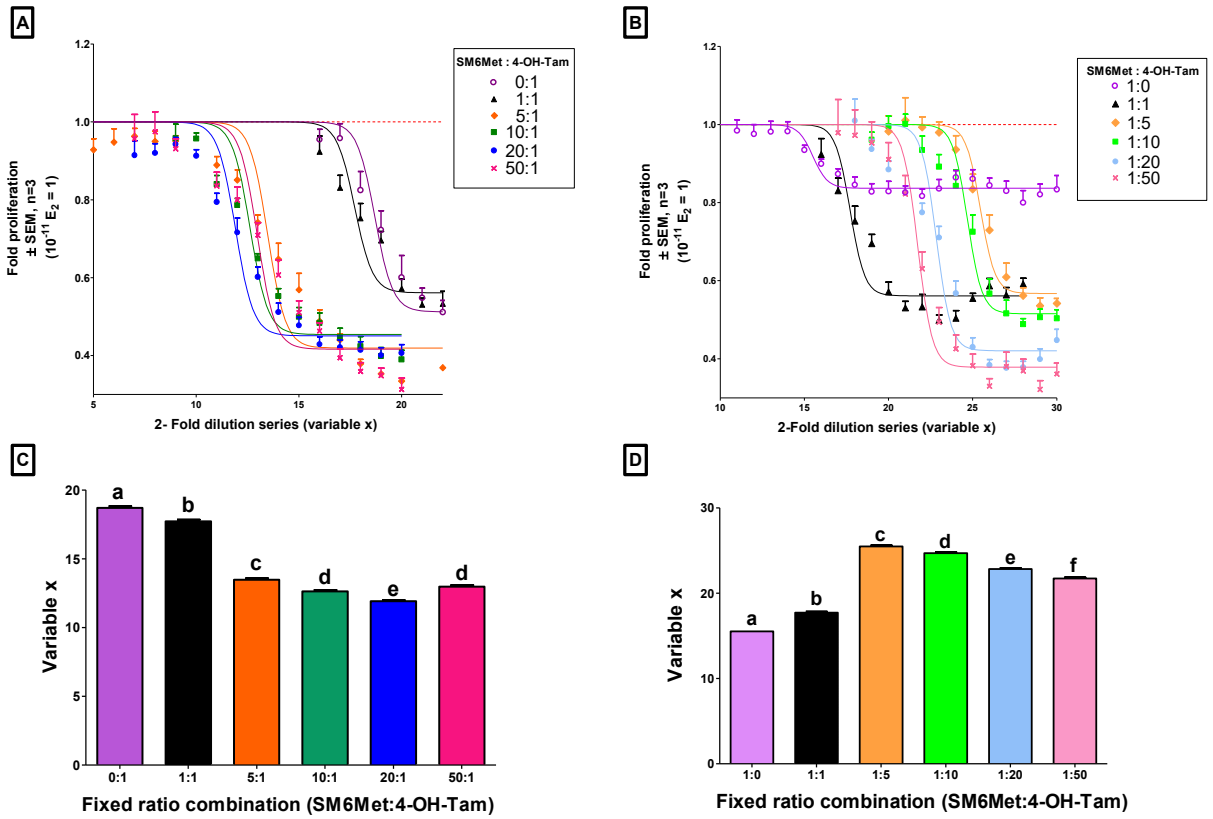

Figure 3.9: Dose response curves illustrating the effect of the combination treatment of SM6Met with 4-OH-Tam in different IC_{50}:IC_{50} ratios. MCF-7BUS cells were treated with combinations with a higher ratio towards SM6Met **(A)** and combinations with a higher ratio towards 4-OH-Tam **(B)** using a 2-fold dilution series of each combination ratio in the presence of 10^{-11}M E_2 for a period of seven days wherein there were two retreatments. Thereafter, MTT solution was added to the cells and after a 4hr incubation the medium was removed and the formazan crystals that formed through metabolism was dissolved in isopropanol. The number of viable cells were measured as absorbance to generate dose response curves using non-linear regression fitting to determine the potency and efficacy values of each combination. Statistical analysis was performed using One-way ANOVA with Tukey's multiple comparisons test as post-test, where different letters indicate statistical significance ($P<0.05$). Average ± SEM is of three independent biological experiments done in quadruplicate.

Table 5: Summary of the concentrations (µg/ml) of SM6Met and 4-OH-Tam for each combination ratio, as determined by non-linear regression analysis, at effective levels of 50%, 75% and 90% in Figure

Ratio of SM6Met:4-OH-Tam	Concentration in µg/ml at the ED_{50} ±SEM		Concentration in µg/ml at the ED_{75} ±SEM		Concentration in µg/ml at the ED_{90} ±SEM		Efficacy[c]
	SM6Met (a^a)	4-OH-Tam (b^b)	SM6Met (a^a)	4-OH-Tam (b^b)	SM6Met (a^a)	4-OH-Tam (b^b)	
1:0 (SM6Met alone)	1.66×10^{-3} ± 0.23	-	9.95×10^{-4} ± 0.61	-	4.97×10^{-4} ± 0.94	-	16.4%
0:1 (4-OH-Tam alone)	-	5.32×10^{-7} ± 0.12	-	2.66×10^{-7} ± 0.34	-	1.25×10^{-7} ± 0.50	48.8%
1:1	7.51×10^{-3} ± 0.12	2.66×10^{-7} ± 0.12	3.54×10^{-3} ± 0.33	1.25×10^{-7} ± 0.33	1.55×10^{-3} ± 0.50	5.47×10^{-8} ± 0.50	43.9%
1:5	1.70 ± 0.14	6.01×10^{-5} ± 0.14	9.05×10^{-1} ± 0.28	3.20×10^{-5} ± 0.28	4.81×10^{-1}± 0.44	1.70×10^{-5} ± 0.44	43.3%
5:1	4.14×10^{-4} ± 0.11	1.47×10^{-8} ± 0.11	1.24×10^{-4} ± 0.21	4.40×10^{-9} ± 0.21	2.94×10^{-5} ± 0.35	1.04×10^{-9} ± 0.35	58%
1:10	9.62×10^{-1} ± 0.11	3.40×10^{-5} ± 0.11	4.53×10^{-1} ± 0.24	1.60×10^{-5} ± 0.24	1.98×10^{-1} ± 0.38	7.01×10^{-6} ± 0.38	48.5%
10:1	2.21×10^{-4} ± 0.09	7.82×10^{-9} ± 0.09	7.60×10^{-5} ± 0.29	2.69×10^{-9} ± 0.29	2.59×10^{-5} ± 0.44	9.16×10^{-10} ± 0.44	54.6%
1:20	2.55×10^{-1} ± 0.11	9.01×10^{-6} ± 0.11	8.49×10^{-2} ± 0.27	3.00×10^{-6} ± 0.27	3.01×10^{-2} ± 0.42	1.06×10^{-6} ± 0.42	57.9%
20:1	1.31×10^{-4} ± 0.08	4.64×10^{-9} ± 0.08	4.83×10^{-5} ± 0.30	1.71×10^{-9} ± 0.30	1.55×10^{-5} ± 0.46	5.50×10^{-10} ± 0.46	54.9%
1:50	1.20×10^{-1} ± 0.13	4.25×10^{-6} ± 0.13	6.01×10^{-2} ± 0.27	2.13×10^{-6} ± 0.27	3.01×10^{-2} ± 0.42	1.06×10^{-6} ± 0.42	62.1%
50:1	2.76×10^{-4} ± 0.10	9.78×10^{-9} ± 0.10	9.67×10^{-5} ± 0.35	3.42×10^{-9} ± 0.35	2.24 x1 0^{-5} ± 0.58	7.94×10^{-10} ± 0.58	58.4%

[a]Variable representing the concentration of SM6Met used in the specified combination ratio that elicits the 50%, 75% or 90% inhibitory effect calculated from the dose response curve depicted in Figure.

[b]Variable representing the concentration of 4-OH-Tam used in the specified combination ratio that elicits the half maximal 50%, 75% or 90% inhibitory effect calculated from the dose response curve depicted in Figure.

[c]Efficacy shown as % inhibition of E_2-induced proliferation.

3.3.3.2.2 The combinatorial effects of SM6Met with 4-OH-Tam on breast cancer cell proliferation is synergistic

The degree of synergism was determined using the interaction index equation,

$$\gamma = \frac{a}{A} + \frac{b}{B} \quad (1)$$

where the interaction index (γ) is equal to the sum of the concentration of SM6Met [a] and 4-OH-Tam [b] at the IC_{50}, IC_{70} or IC_{90} point of the selected combination ratio, divided by the IC_{50}, IC_{75} or IC_{90} concentration of (A) SM6Met alone (1:0) and (B) 4-OH-Tam alone (0:1), (Table 4). If the combination is synergistic, the index will be less than one ($\gamma < 1$), if additive it will be equal to one ($\gamma = 1$), while if it is antagonistic the index will be greater than one ($\gamma > 1$) (76). In layman's terms, antagonism signifies drugs acting against each other, synergism signifies drugs working together and additivity is the summation of the individual drug effects also referred to as the zero-interactive state (77).

Table 6: Summary of the interaction index for each combination ratio, at effective levels 50%, 75% and 90%

Ratio of SM6Met:4-OH-Tam	Interaction Index at 50% effect level (γ_{50})	Interaction Index at 75% effect level (γ_{75})	Interaction Index at 90% effect level (γ_{90})
1:50	80.28	68.41	69.04
1:20	170.55	96.60	69.04
1:10	643.43	515.43	454.47
1:5	1137.07	1029.85	1103.81
1:1	5.02	4.03	3.56
5:1	0.28	0.14	0.07
10:1	0.15	0.09	0.06
20:1	0.09	0.05	0.04
50:1	0.18	0.11	0.05

The interaction index values calculated and summarized in Table 6 was used to create an interaction index plot, a convenient and simple graphic representation of the interaction index (Figure). The combinations with a higher ratio towards SM6Met (5:1, 10:1, 20:1 and 50:1) showed an interaction index less than one at all three selected effect levels, whereas the 1:1 combination of SM6Met with 4-OH-Tam and all of combinations with higher ratios towards 4-OH-Tam (1:5, 1:10, 1:20 and 1:50) displayed an interaction index greater than one at all three selected effect levels, thereby indicating that increasing the concentration of

SM6Met in the combination synergizes the effect of 4-OH-Tam, while increasing the concentration of 4-OH-Tam in the combination antagonizes the effect of SM6Met.

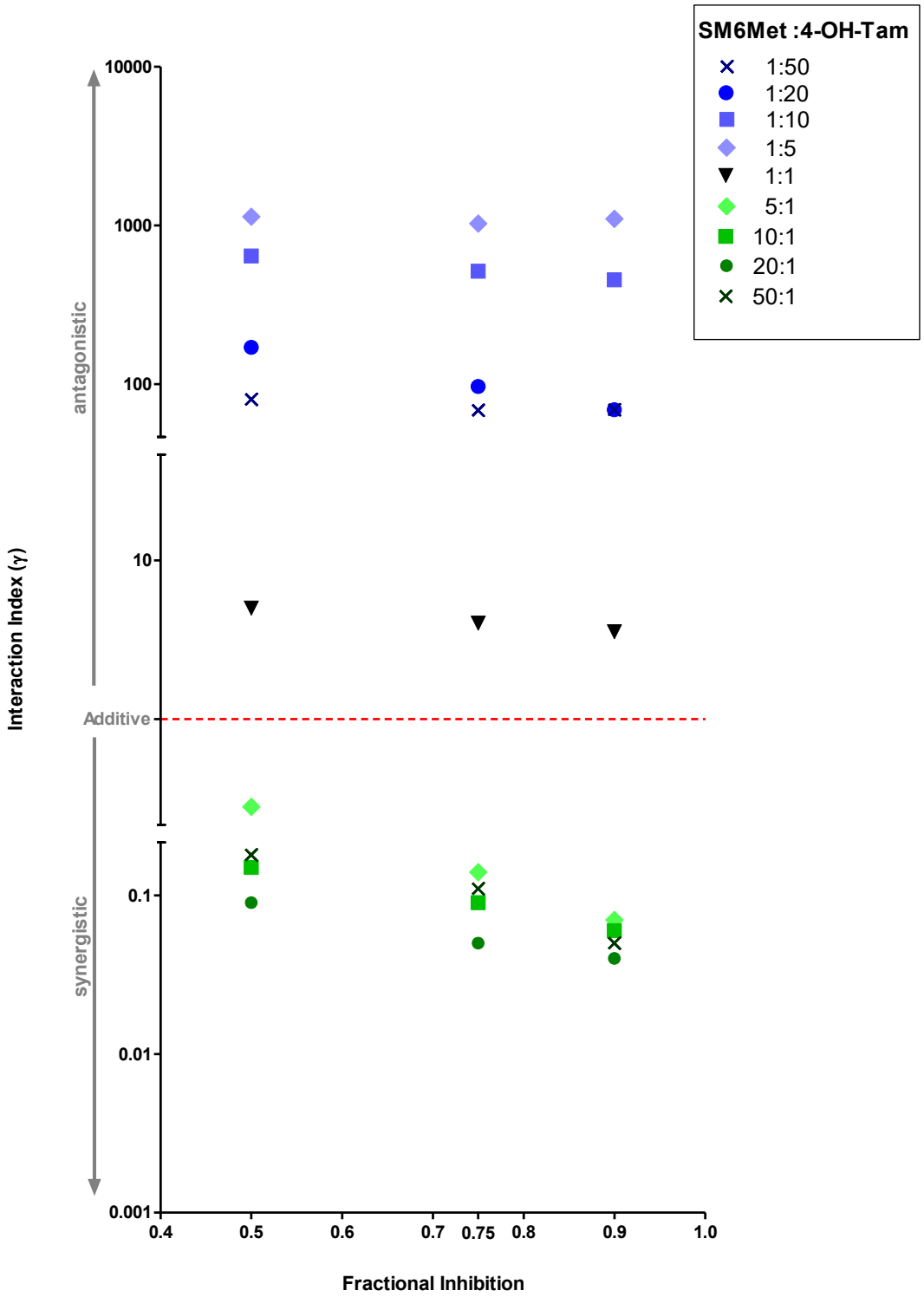

Figure 3.10: Interaction index plot for the SM6Met:4-OH-Tam ratio combinations at 50%, 75% and 90% inhibition of breast cancer cell proliferation. The dose of SM6Met in combination (a) divided by the dose of SM6Met alone (A) plus the dose of 4-OH-Tam in combination (b) divided by the dose of 4-OH-Tam alone (B) at the selected effect level equals the interaction index ($\boldsymbol{\gamma}$). If the combination is synergistic, the index will be less than one ($\boldsymbol{\gamma < 1}$), if additive it will be equal to one ($\boldsymbol{\gamma = 1}$), while if it is antagonistic the index will be greater than one ($\boldsymbol{\gamma > 1}$).

The combinations may be listed in the order of increasing synergism as follows: 5:1 < 50:1 < 10:1 < 20:1 at 50% and 75% inhibition and 5:1 < 10:1 < 50:1 < 20:1 at 90% inhibition. The combinations may be listed in the order of increasing antagonism as follows: 1:1 < 1:50 < 1:20 < 1:10 < 1:5 at 50% and 75% inhibition; and 1:1 < 1:50 = 1:20 < 1:10 < 1:5 at 90% inhibition (Figure and Table 6). In summary, the combination ratio of SM6Met:4-OH-Tam of 20:1 displayed the lowest interaction index at all the effect levels thereby making it the combination ratio with the highest degree of synergism.

3.4 Discussion

The adverse side-effects together with the high instances of resistance encountered with the use of SOC hormone therapy for breast cancer treatment such as SERMs, like tamoxifen, and SERDs, like fulvestrant (78), have led to the search for healthier, more natural compounds for breast cancer prevention and treatment as well as for new strategies, like multi-targeted therapy also known as combination therapy. Many of these strategies involve combining natural products, like tea extracts, with conventional hormone therapies (41–43).

3.4.1 Evaluation of the ER subtype selectivity of the test panel

In this study we validated the subtype selectivity of SM6Met in two cell models, that don't express endogenous ER, by comparing it to known ER subtype agonists and antagonists including the natural ligand, E_2 (summarized in Table 2 and Table 3). Transfection of the ER subtypes into the two cell models, the HEK293 and the COS-1 cell line, allowed for evaluation of agonism and antagonism of the ER subtypes separately using the classical ERE transactivation model.

E_2, in correspondence with previous studies, acted as a full agonist of both receptor subtypes with a higher potency via ERα than via ERβ in both HEK293 and COS-1 cell lines (50, 52, 79, 80). In contrast, Barkhem *et al*. (81), showed that E_2 displayed a higher potency via ERβ than via ERα in the HEK293 cell line.

As expected the two SOC treatments, fulvestrant and 4-OH-Tam, both acted as antagonists of E_2-induced transactivation of ERα and ERβ in both cell lines. However, the inverse agonism of 4-OH-Tam via ERα in the HEK293 cell line was an unexpected result, as a conscious effort was made to limit or remove any basal steroid levels by performing the assay in phenol red free medium with DS-FCS. To our knowledge this inverse agonist effect has not been described in previous studies and this phenomenon may be ascribed to the use of different experimental conditions, cell lines, plasmids, response elements and estrogen receptor concentrations than that used by other studies found in literature or to the presence or absence of certain co-regulators in different cell lines.

MPP antagonized the effect of E_2 via ERα in the HEK293 cell line with the same potency as MPP antagonized E_2-induced ERE-promoter reporter activity via ERα in the study of Zhou *et al.* (82), using HEC-1 cells. As expected liquiritigenin showed agonist effects via ERβ, however, the potency with which it activated the ERE-luciferase activity via ERβ differed from the study done by Mersereau *et al.* (66). In the current study, the potency of liquiritigenin via ERβ in the HEK293 cells was significantly ($P<0.001$) 136.8-fold higher than via ERβ in the COS-1 cell line, moreover it was 492.1-fold higher than the potency (36.5

nM) for liquiritigenin via ERβ found by Mersereau *et al.* (66), in transiently transfected human osteosarcoma U2OS cells. Although liquiritigenin did not display any agonist effects via ERα in either of the two cell lines, some studies have shown liquiritigenin to be a partial ERα agonist at high concentrations equivalent to what was used in the current study (Suppl. Fig. S4) (83).

In correspondence to a study done by Visser *et al.* (52), SM6Met displayed agonist effects via ERβ and antagonistic effects via ERα, however, in contrast to the study by Visser *et al.*, dose response curves were established and potency and efficacy values could be determined in the current study.

In summary, from the ERE-containing promoter reporter studies we were able to validate the agonist/antagonist profiles of all the test compounds, as well as the extract, SM6Met. With the knowledge that ERα is associated with increased breast cancer cell proliferation, while ERβ has been associated with tumour suppressor characteristics by inhibiting the effects of ERα (10, 12, 84, 85), the subtype selective properties of SM6Met may be of physiological importance as it could possibly enhance the prevention of excessive cell growth associated with cancer development (86).

3.4.2 Evaluation of the effect of the test panel on E_2-induced breast cancer proliferation

A cascade of events that elicits an estrogenic or anti-estrogenic response, is initiated by a ligand binding to the ER subtypes. Therefore, transactivation of an ERE-containing promoter reporter element as well as induction or repression of cell proliferation are both endpoints used to identify and evaluate the estrogenic or anti-estrogenic potential of a compound or extract (50). We, therefore, set out to determine the effects SM6Met on a more physiologically relevant model (MCF-7BUS cell line), where the ER subtypes are co-expressed (Figure) in order to evaluate the effect of SM6Met on breast cancer cell proliferation and compare its effects to the SOC therapies as well as to the commercially available ER subtype selective ligands.

E_2 was the only compound from the test panel that induced breast cancer cell proliferation, with a potency (7.6×10^{-7}µg/ml) more or less similar to the potency (2.79×10^{-7}µg/ml) determined by Verhoog *et al.* (87), both about 10-fold more potent than the potency (2.59×10^{-6}µg/ml) determined by Visser *et al.* (52). The proliferative results suggest that E_2 is ER dependant as it was antagonized by the ER down-regulator, fulvestrant in the current study.

It was not surprising that the SOC treatment, 4-OH-Tam, which inhibited E_2 induced ERE-luc transactivation via ERα and ERβ, was able to inhibit E_2 induced breast cancer cell proliferation, yet, fulvestrant was able to do so at a higher efficacy and potency than 4-OH-Tam (Figure and Table 4). The potency with which 4-OH-Tam (3.1×10^{-3}µg/ml) inhibited the E_2 induced proliferation of the MCF7-BUS cells in the current study was found to be about 1626-fold higher than the potency (13.13µM or 5.04µg/ml) of 4-OH-Tam determined by Roberts *et al.* (88), using MCF-7 and T47D breast cancer cells. The potency of fulvestrant (1.1×10^{-4}µg/ml) in the current study was also found to be about 49-fold higher than the potency (9nM or 5.46×10^{-3}µg/ml) determined by Joseph *et al.* (89).

Interestingly, MPP, as well as fulvestrant, reduced breast cancer cell proliferation in the absence and presence of E_2 and although no literature was found to support the aforementioned result for MPP, a study by Joseph *et al.* (89) demonstrated the same inverse agonist effect of fulvestrant on breast cancer cell proliferation. In the presence of E_2, liquiritigenin was able to inhibit E_2 induced breast cancer cell proliferation at certain concentrations (2.6×10^{-9}µg/ml to 3.9×10^{-5}µg/ml), however, the potency and efficacy of liquiritigenin could not be determined as a dose-response curve could not be established (Suppl. Fig. S8E). With regard to transactivation, both SM6Met and liquiritigenin displayed agonism via ERβ, however, unlike SM6Met, liquiritigenin had no effect on transactivation via ERα, thereby, suggesting that agonism through ERβ alone is not enough to inhibit breast cancer cell proliferation to a degree that is comparable to current SOC therapies.

In contrast to the study done by Visser *et al.* (52) showing inhibition of E_2 induced breast cancer cell proliferation only at certain concentrations (0.98, 9.8 and 98000µg/ml), in the current study SM6Met was able to inhibit E_2 induced breast cancer cell proliferation in a dose dependant manner allowing for the determination of the potency and efficacy of SM6Met. However, SM6Met was not as effective or potent as either 4-OH-Tam or fulvestrant. SM6Met as a monotherapy was therefore not able to compete with the efficacy or potency of current SOC therapies (fulvestrant and 4-OH-Tam). Although SM6Met was the least effective of all the antagonists of E_2 induced breast cancer cell proliferation, it proved to be more potent than the ERα selective antagonist, MPP. This suggests that a compound or extract that combines ERα antagonist and ERβ agonist properties may be more beneficial for breast cancer treatment or prevention than a compound that selectively antagonizes only ERα (MPP) or a compound that acts as a selective ERβ agonist (Liq).

3.4.3 Proof of concept that a combination of an ERα antagonist and ERβ agonist is more effective in preventing E_2 induced breast cancer cell proliferation than an ERα antagonist or ERβ agonist on their own

Nutraceuticals are generally less potent than synthetic drugs as synthetic drugs are the pure, highly concentrated active ingredient of an extract or natural compound that has been chemically replicated and mass produced. A recent study showed that the desirable estrogenic effects of SM6Met could not be retained or significantly enhanced by fractionation (90). In addition, another limitation of natural compounds and extracts include differences in composition of batches harvested due to variables in the environment (91–93). Therefore, the checkerboard assay was used to combine an ERα selective antagonist (MPP) with an ERβ selective agonist (liquiritigenin) to mimic the SERSM characteristics of SM6Met and to validate the principle that a compound with the subtype selective properties of SM6Met would be more advantageous in inhibiting E_2 induced breast cancer cell growth than generally used SERMs, like tamoxifen, or an ERα selective antagonist (MPP) or an ERβ selective agonist (liquiritigenin) on their own.

With regard to inhibition of E_2 induced breast cancer cell proliferation, combining the ERα antagonist, MPP, with the ERβ agonist, liquiritigenin, resulted in a higher reduction of E_2 induced breast cancer proliferation in comparison to the ER selective ligands alone. The most effective combination (10^{-7}M liquiritigenin together with 10^{-6}M MPP) was not only more effective than 10^{-6}M MPP alone and 10^{-7}M liquiritigenin alone, but was also more effective than the SOC therapies, 4-OH-Tam and fulvestrant on their own (Table 4),

thereby suggesting that the ideal ER subtype selective properties of SM6Met can be replicated and a purer, highly concentrated synthetic drug or nutraceutical with the same SERSM characteristics could possibly be synthesized. To our knowledge the combination of a selective ERα antagonist, like MPP, with a selective ERβ agonist, like liquiritigenin, has not previously been investigated and results pertaining to the effects of this combination are novel.

3.4.4 Evaluation of SM6Met and 4-OH-Tamoxifen as combined therapy to inhibit breast cancer cell proliferation

Drug combination studies are becoming increasingly more popular in the field of cancer research in an attempt to lower side-effects related to current therapies and to lower the risk of resistance by lowering the doses of drugs needed to elicit the desired effects (73, 94). After validating the beneficial effects of ER subtype selective treatment in lowering breast cancer growth, I wanted to evaluate the potential of SM6Met to be used in combination with other SOC treatments as a multi-targeted approach to lowering breast cancer growth.

The checkerboard assay showed that the degree to which E_2 induced breast cancer cell proliferation was inhibited was increased by combining SM6Met with 4-OH-Tam, however enhanced effectiveness does not necessarily mean that the drug combination is synergistic (73). Therefore, the synergistic potential of SM6Met in combination with 4-OH-Tam was evaluated using the fixed ratio isobolar method as described by Tallarida *et al.* (74, 75). This study, demonstrated for the first time that the *C. subternata* extract, SM6Met, synergistically promoted tamoxifen-induced antagonism of E_2 induced breast cancer proliferation.

The results showed that increasing the concentration of SM6Met in the combination ratio of SM6Met:4-OH-Tam resulted in an increase in potency up to a point where the endogenous ERs possibly became saturated (ratio of 50:1) and the potency could not increase further (FigureC). Increasing the concentration of 4-OH-Tam in the combination ratio of SM6Met:4-OH-Tam resulted in a decrease in potency at the ratio of 1:1 and 1:5, however, when 4-OH-Tam is increased more than five times in relation to SM6Met (1:10, 1:20 and 1:50), the potency increases slightly, but never reaching the same potency as that of the combination ratio 1:1. This strange biphasic trend in potency changes could be due to the high concentration of 4-OH-Tam present in the ratios 1:10, 1:20 and 1:50, which could possibly completely outcompete SM6Met, thereby shifting the response to resemble the effects of 4-OH-Tam as monotherapy.

The degree of synergism was determined using the interaction index (γ) (74, 75). If the combination ratio is synergistic its interaction index value will be less than one ($\boldsymbol{\gamma < 1}$), if additive it will be equal to one ($\boldsymbol{\gamma = 1}$), while if it is antagonistic the index will be greater than one ($\boldsymbol{\gamma > 1}$). The combination ratio 20:1 was the combination of SM6Met:4-OH-Tam with the lowest interaction index, therefore, the highest degree of synergism. We used the interaction index plot instead of the conventional isobologram to simplify the graphical representation of the data in addition to showing the relation between the different effect levels.

In regards to anti-cancer therapies, synergism ($\gamma < 1$) at high effect levels is better than synergism at low effect levels, for example a combination therapy that is synergistic at the low effect level of 50% means that only 50% of the concentration range used in the dose response curve will be synergistic, whereas a combination therapy that is synergistic at a high effect level of 90% will be synergistic at 90% of the concentration range of the dose response curve. Therefore, the combination ratio 20:1 not only has the lowest interaction index, but it is synergistic at all the effect levels (50%, 75% and 90%) tested.

Other studies have also reported synergistic effects of plant-derived products in combination with 4-OH-Tam. Examples include a study by Yaacob *et al*. (42), who used the Chou-Talalay non-constant ratio drug combination method and showed synergistic inhibition of MCF-7 and MDA-MB-231 breast cancer cell growth by the combination of a bioactive subfraction of *Strobilanthes crispus* leaves (SCS - a shrub originally from Madagascar) and tamoxifen with combination index values of 0.32 – 0.40 for MCF-7 cells and 0.29 – 0.52 for MDA-MB-231 cells at 84 – 97% effect levels. A study by Chisholm *et al.* (43) where the synergistic cytotoxic effects of epigallocatechin gallate (the most common catechin found in green tea) in combination with tamoxifen on MDA-MB-231 breast cancer cells was shown and a study by Samadi *et al.* (95) that showed synergistic inhibition of proliferation and induction of apoptosis in MDA-MB-231 and H1299 cells by the combination of vinblastine (isolated from the flowering Madagascan plant, *Catharanthus roseus*) with tamoxifen.

Due to the various theories, hypotheses, approaches and models used, it is hard to compare the claimed synergistic results of the studies previously mentioned with the results obtained in the current study. Although all three studies claim synergism, the studies by Chisholm *et al*. (43) and Samadi *et al*. (95) only show enhanced effectiveness for the various combinations and as described by Chou *et al*. (69, 73) enhanced effectiveness does not necessarily mean that the drug combination is synergistic. The most recent methods to determine synergy describe synergism as a measure of the degree of enhancement or reduction in potency and not effectiveness (73, 76). Without a standardized method of analysis, unsubstantiated or faulty claims of synergism are unescapable. However, the study by Yaacob *et al.* (42) did establish combination index (CI) values, which is comparable to the interaction index calculated in the current study, but for effect levels (84-97%), which differ from those of the current study (50%,75% and 90%). Nonetheless, the best interaction index (0.04) achieved in the current study by the 20:1 ratio of SM6Met:4-OH-Tam at the 90% effect level is substantially greater than the best combination index (0.32) achieved by Yaacob *et al.* (42) in the MCF-7 cell line.

In summary, the presence of multiple compounds in natural agents such as SM6Met may provide the advantage of acting on multiple pathways that control the process of cancer development and progression and highlight the importance of natural agents in the research, understanding and development of combinatorial therapies for cancer prevention and treatment.

3.4.5 In conclusion

The results of this study may be assessed in terms of the potential of SM6Met, a SERSM, as an alternative treatment for breast cancer, either as monotherapy or in combination with current SOCs like 4-OH-Tam. With regard to SM6Met as monotherapy, it is clear that SM6Met could not, in terms of efficacy or potency,

compete with the SOC treatments like 4-OH-Tam and fulvestrant and future work should concentrate on enhancing the effects of SM6Met or recreating its ER subtype selective characteristics synthetically.

However, the most exciting outcome of this study is the two novel findings with regard to combination therapies. Firstly, the most effective combination of the ERα antagonist, MPP, with the ERβ agonist, liquiritigenin, displayed 24.5%, 33.5% and 17.5% higher inhibition of E_2-induced breast cancer cell proliferation than MPP and the SOC therapies, 4-OH-Tam and fulvestrant, respectively, thereby validating the concept that a mixture displaying both ERα antagonist and ERβ agonist properties is not only more effective than ER subtype selective compounds on their own at reducing E_2-induced breast cancer cell proliferation, but also more effective than some SERMs and SERDs on their own. These results therefore show that extracts like SM6Met that contain compounds that selectively modulate the ER subtypes show promise for the development of anti-cancer agents.

Secondly, the observation that SM6Met synergized the anti-proliferative effects of 4-OH-Tam on E_2-induced breast cancer proliferation with the combination ratio of 20:1 displaying the highest degree of synergism. Subsequently in combination with SM6Met about 20 times lower concentrations of 4-OH-Tam are needed to elicit the same effect as 4-OH-Tam alone and therefore such combined treatments could not only potentially prevent or delay the onset of resistance, but also potentially lower or delay the onset of adverse side effects associated with tamoxifen use (42, 43, 75). Further work should be done to clarify the mechanism with which SM6Met synergizes the effects of 4-OH-Tam as well as on the potential synergistic effects of SM6Met in combination with other SOC therapies like other SERMs, SERDs or chemotherapies.

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3.6 Supplementary Figures

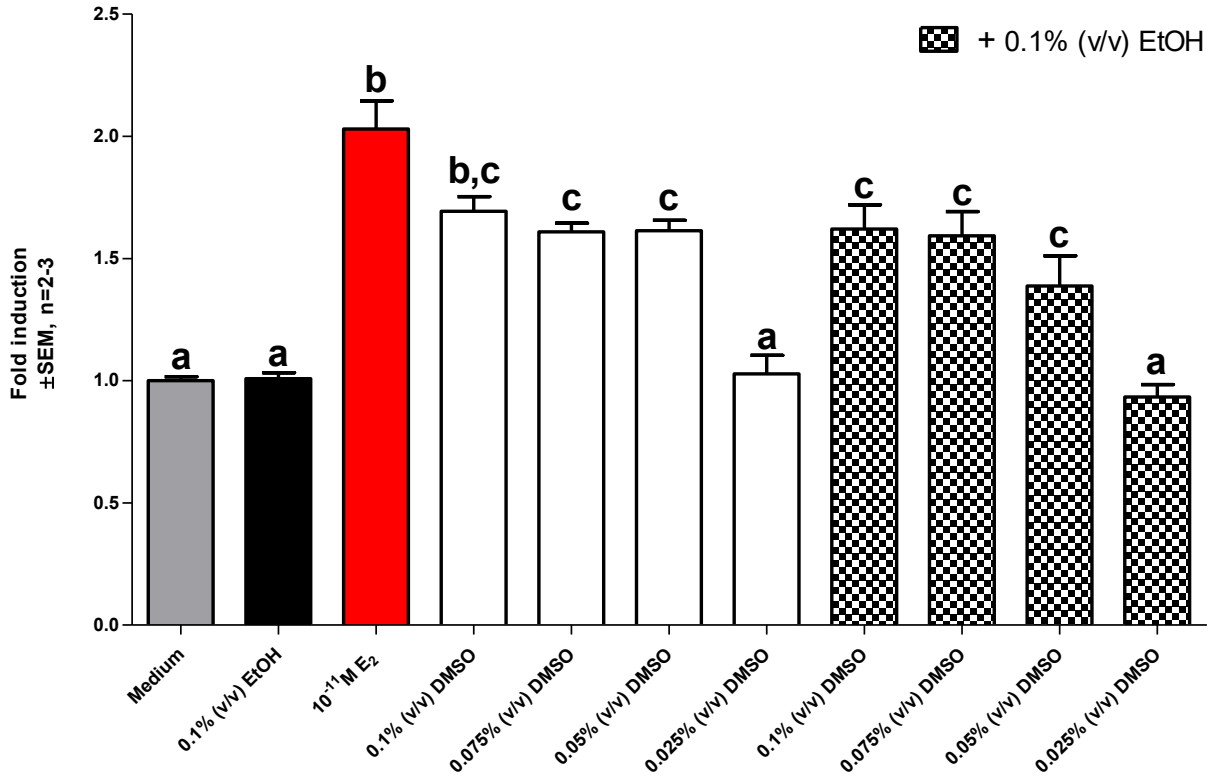

Figure S1: Evaluation of the proliferative potential of the solvents used to prepare stock solutions of the test panel, which was used for induction in all experimental work. The MCF-7BUS cell were withdrawn from steroids for a week before plating, by changing the growth medium to treatment medium. Thereafter, MCF-7BUS cells were induced with the three negative solvent controls including (1) treatment medium, (2) 0.1(v/v) EtOH in treatment medium and (3) 0.025% (v/v) DMSO in treatment medium together with the positive control, 10^{-11}M E_2, for a period of seven days, wherein there were two retreatments. Thereafter, MTT solution was added to the cells and after a 4hr incubation the medium was removed and the formazan crystals that formed through metabolism was dissolved in isopropanol. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test as post-test, where different letters indicate statistical significance ($P<0.05$). Average ± SEM is of two to three independent biological experiments done in duplicate.

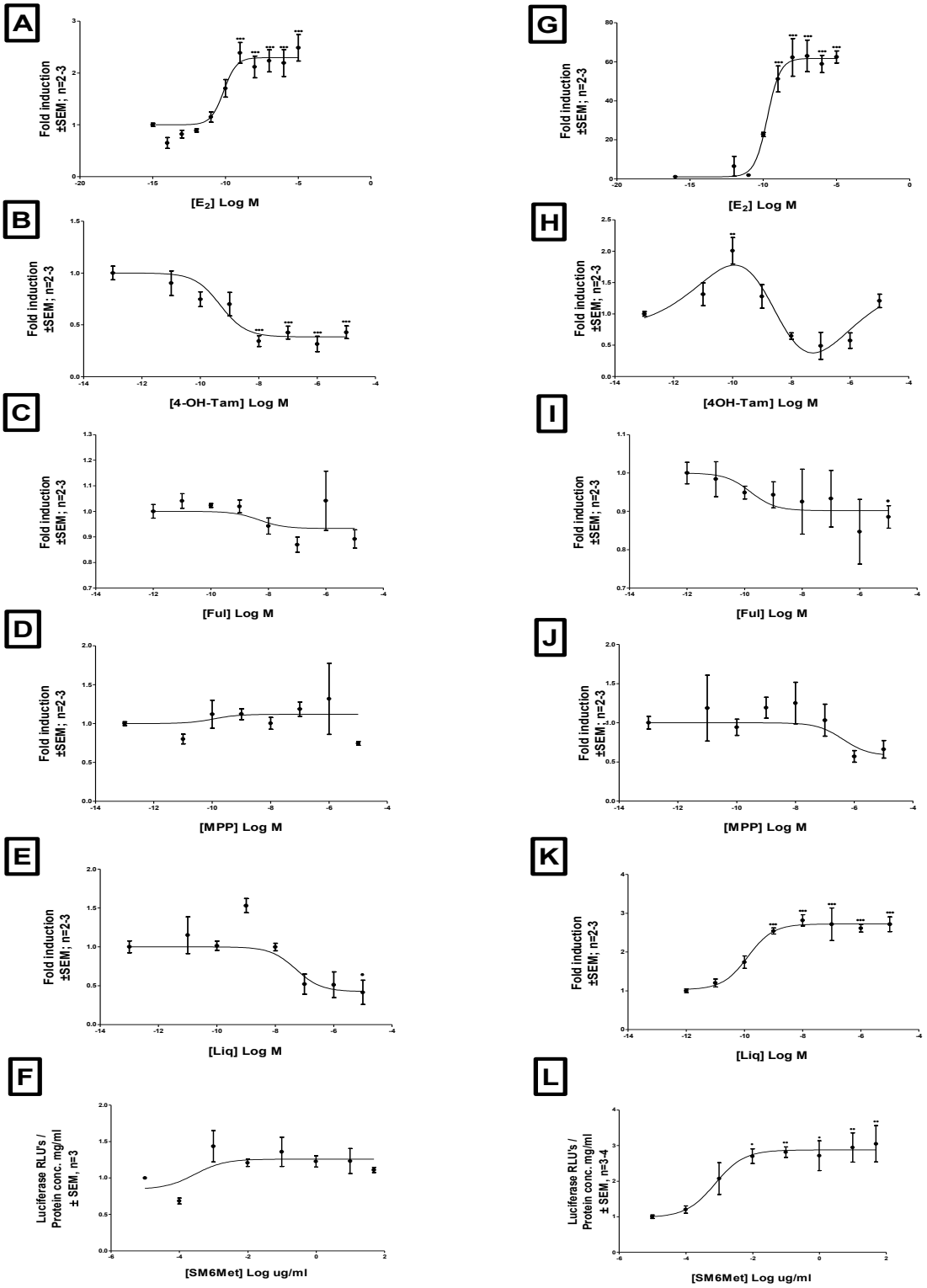

Figure S2: Evaluation of subtype selective agonism of all the test panel by ERE-containing promoter reporter activity induced in ERα and ERβ transfected HEK 293 cells by the different compounds and extract. HEK 293 cells were transiently transfected with 150ng of pSG5-hERα (A-F) or pSG5-hERβ (G-L) together with an ERE-containing promoter reporter constructs (ng) and left for 24hrs. After transfection, the cells were re-plated into sterile 24 well plates and treated 24hrs later with E_2 (A, G), 4-OH-Tam (B, H), fulvestrant (C, I), MPP (D, J), liquiritigenin (E, K) and SM6Met (F, L) in a dose dependant manner for 24hrs. Subsequent to the 24hr induction with the test panel, the cells were lysed using passive lysis buffer. Luciferase activity was measured and normalised to protein content which was determined using a Bradford assay. One-way ANOVA with Tukey's multiple comparisons test as post-test, showing statistical difference to the solvent control ($^{\bullet}$, P<0.05; $^{\bullet\bullet}$, P<0.01, $^{\bullet\bullet\bullet}$, P<0.001) was performed for statistical analysis. The average ±SEM is of two to three independent biological experiments done in triplicate.

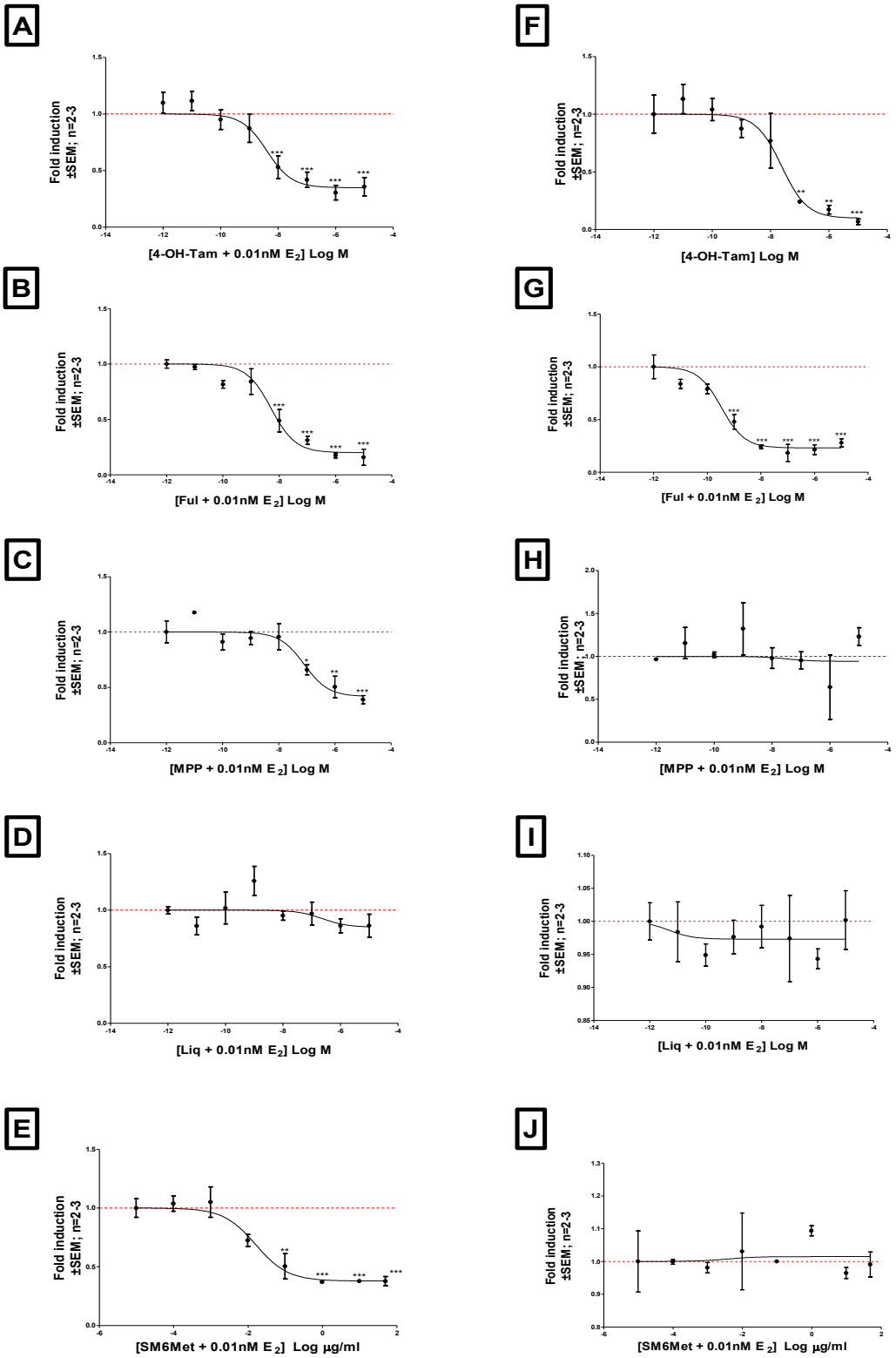

Figure S3: Evaluation of subtype selective antagonism of the test panel by ERE-containing promoter reporter activity induced in ERα and ERβ transfected HEK 293 cells by the different compounds and extract. HEK 293 cells were transiently transfected with either pSG5-hERα (A-E) or pSG5-hERβ (F-J) together with an ERE-containing promoter reporter constructs (ng) and left for 24hrs. After transfection, the cells were re-plated into sterile 24 well plates and treated 24hrs later with 4-OH-Tam (A, F), fulvestrant (B, G), MPP (C, H), liquiritigenin (D, I) and SM6Met (E, J), all in the presence of 10^{-11}M E_2, in a dose dependant manner for 24hrs. Subsequent to the 24hr induction with the test panel, the cells were lysed using passive lysis buffer. Luciferase activity was measured and normalised to protein content which was determined using a Bradford assay. One-way ANOVA with Tukey's multiple comparisons test as post-test, showing statistical difference to 10^{-11}M E_2 (*, P<0.05; **, P<0.01, ***, P<0.001) was performed for statistical analysis. The average ±SEM is of two to three independent biological experiments done in triplicate.

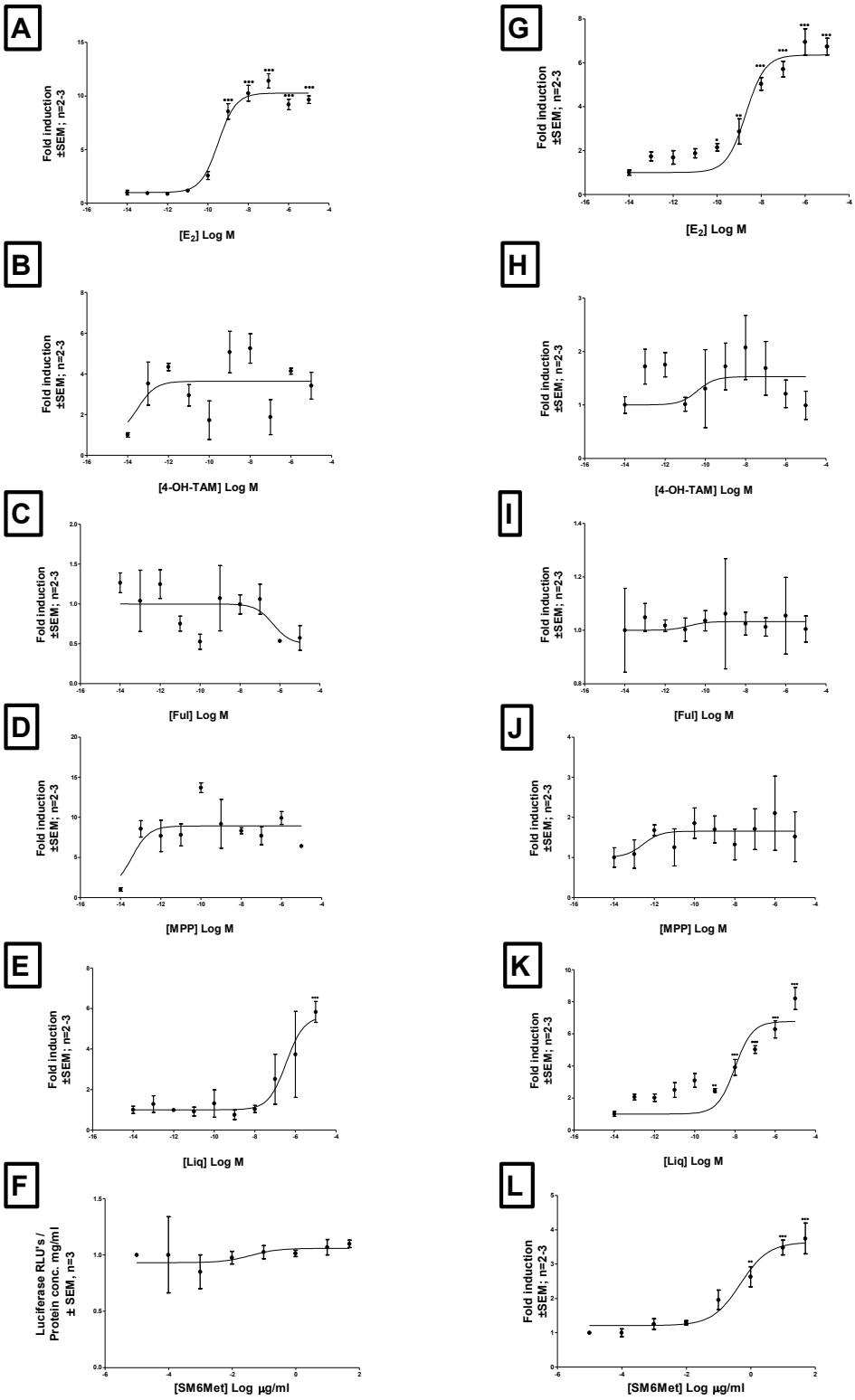

Figure S4: Evaluation of subtype selective agonism of the test panel by ERE-containing promoter reporter activity induced in ERα and ERβ transfected COS-1 cells by the different compounds and extract. COS-1 cells were transiently transfected with 150ng of pSG5-hERα (A-F) and pSG5-hERβ (G-L) together with an ERE-containing promoter reporter constructs (ng) and left for 24hrs. After transfection, the cells were re-plated into sterile 24 well plates and treated 24hrs later with E_2 (A, G), 4-OH-Tam (B, H), fulvestrant (C, I), MPP (D, J), liquiritigenin (E, K) and SM6Met (F, L) in a dose dependant manner for 24hrs. Subsequent to the 24hr induction with the test panel, the cells were lysed using passive lysis buffer. Luciferase activity was measured and normalised to protein content which was determined using a Bradford assay. One-way ANOVA with Tukey's multiple comparisons test as post-test, showing statistical difference to solvent control ($^{\bullet}$, $P<0.05$; $^{\bullet\bullet}$, $P<0.01$, $^{\bullet\bullet\bullet}$, $P<0.001$) was performed for statistical analysis. The average ±SEM is of two to three independent biological experiments done in triplicate.

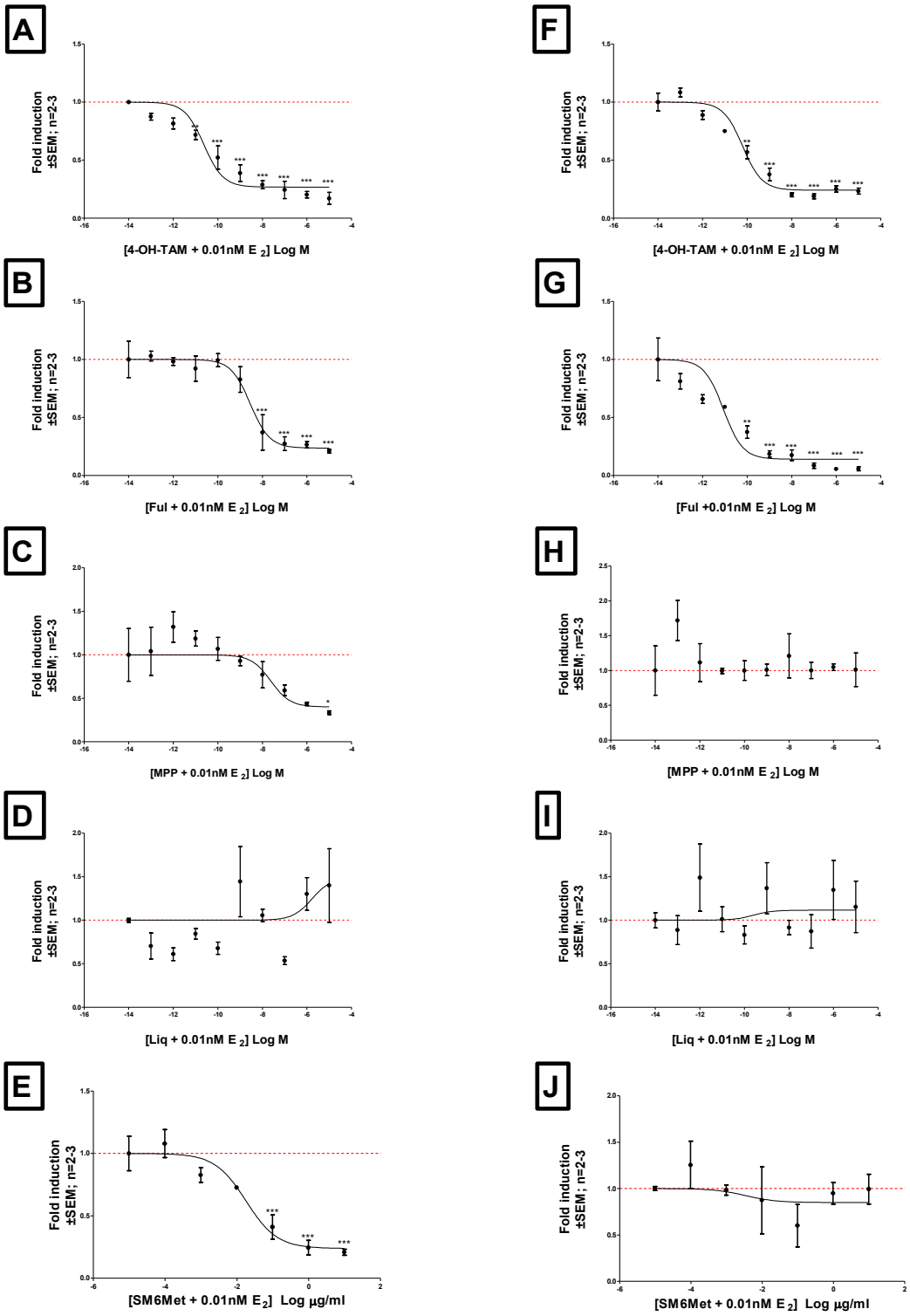

Figure S5: Evaluation of subtype selective antagonism of the test panel by ERE-containing promoter reporter activity induced in ERα and ERβ transfected COS-1 cells by the different compounds and extract. COS-1 cells were transiently transfected with either pSG5-hERα (A-E) or pSG5-hERβ (F-J) together with an ERE-containing promoter reporter constructs (ng) and left for 24hrs. After transfection, the cells were re-plated into sterile 24 well plates and treated 24hrs later with 4-OH-Tam (A, F), fulvestrant (B, G), MPP (C, H), liquiritigenin (D, I) and SM6Met (E, J), all in the presence of 10^{-11}M E_2, in a dose dependant manner for 24hrs. Subsequent to the 24hr induction with the test panel, the cells were lysed using passive lysis buffer. Luciferase activity was measured and normalised to protein content which was determined using a Bradford assay. One-way ANOVA with Tukey's multiple comparisons test as post-test, showing statistical difference to 10^{-11}M E_2 (*, P<0.05; **, P<0.01, ***, P<0.001) was performed for statistical analysis. The average ±SEM is of two to three independent biological experiments done in triplicate.

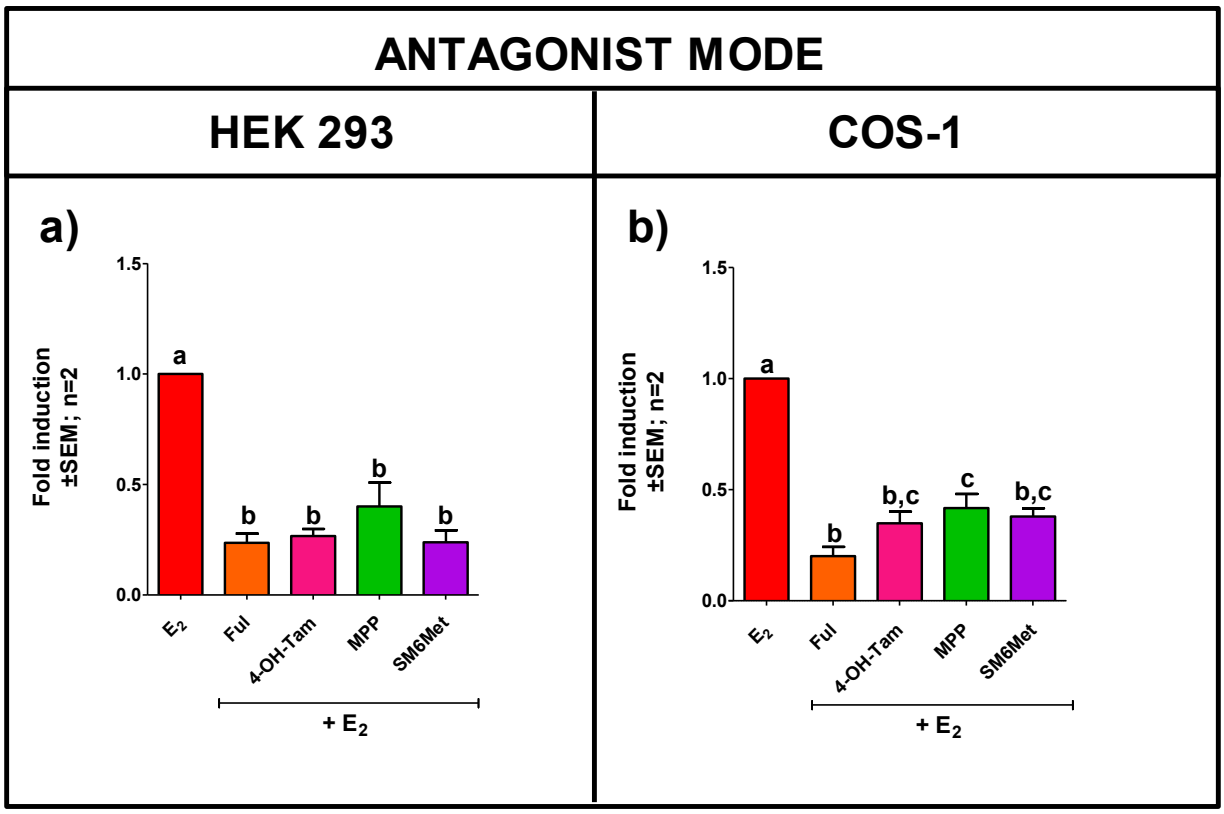

Figure S6: Comparison between the efficacies of the ERα antagonists in the HEK293 and COS-1 cells. HEK293 and COS-1 cells were plated into sterile 10cm^2 tissue culture dishes at a density of 2 x 10^6 cells/dish and transiently transfected with pSG5-hERα together with an ERE-containing promoter reporter construct, 24hrs after plating. Antagonism was tested by treating the cells with 4-OH-Tam, fulvestrant, MPP, liquiritigenin and SM6Met in a dose dependant manner (Figureb & f) in the presence of 10^{-11}M E_2. Subsequent to the 24hr induction with the test panel, the cells were lysed using passive lysis buffer. Luciferase activity was measured and normalised to protein content which was determined using a Bradford assay. Efficacies are expressed as fold values, where the antagonist were normalised to solvent control (E_2 =1). Statistical analysis was performed using One-way ANOVA with Tukey's multiple comparisons test as post-test, where different letters indicate statistical significance (P<0.05). The average ± SEM is of two independent biological experiments done in triplicate.

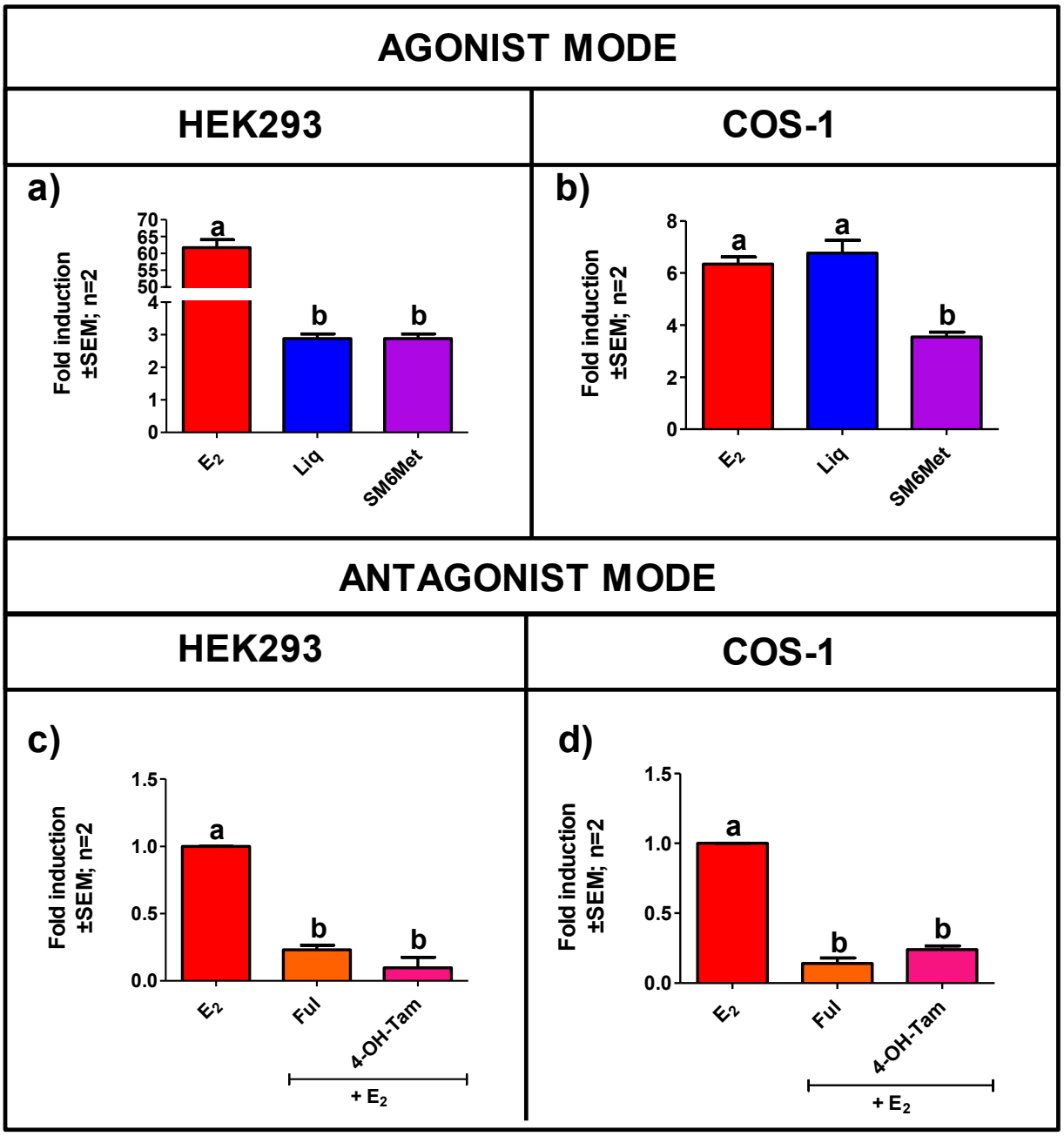

Figure S7: Comparison between the efficacies of the ERβ agonists and antagonists in the HEK293 and COS-1 cells. HEK293 and COS-1 cells were plated into sterile 10cm^2 tissue culture dishes at a density of 2 x 10^6 cells/dish and transiently transfected with pSG5-hERβ together with an ERE-containing promoter reporter construct, 24hrs after plating. To test agonism (a & b) the cells were treated for with E_2, 4-OH-Tam, fulvestrant, MPP, liquiritigenin and SM6Met in a dose dependant manner (Figurea & e) 24hrs after transfection, whereas antagonism (c & d) was tested by treating the cells with 4-OH-Tam, fulvestrant, MPP, liquiritigenin and SM6Met in a dose dependant manner (Figureb & f) in the presence of 10^{-11}M E_2 (set as 1 for antagonism). Subsequent to the 24hr induction with the test panel, the cells were lysed using passive lysis buffer. Luciferase activity was measured and normalised to protein content which was determined using a Bradford assay. Statistical analysis was performed using One-way ANOVA with Tukey's multiple comparisons test as post-test, where different letters indicate statistical significance ($P<0.05$). The average ± SEM is of two independent biological experiments done in triplicate.

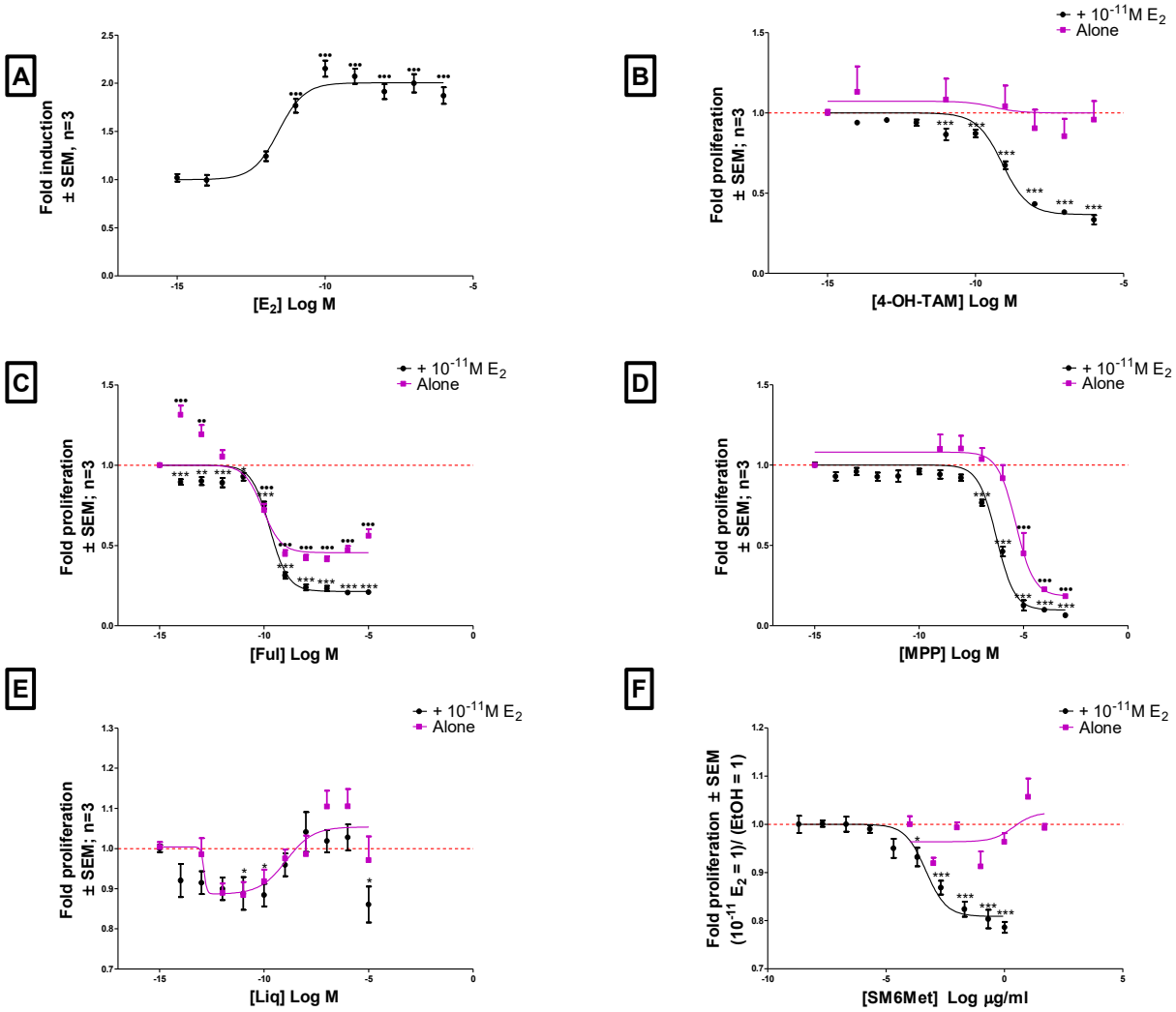

Figure S8: Evaluation of the effects of the test panel on breast cancer cell proliferation in the absence (alone) and presence (+ 10^{-11}M E_2) of E_2, by means of dose response curves. MCF-7BUS cells were treated with a 4-OH-Tam (B), fulvestrant (C), MPP (D), liquiritigenin (E) and SM6Met (F) in the absence and in the presence of 10^{-11}M E_2 (A), in a dose dependent manner for a period of seven days, wherein there were two retreatments on day 3 and 6. Thereafter, MTT solution was added to the cells in order to determine the amount of formazan formed, which indicates the number of viable cells, through metabolism and dissolved in isopropanol. One-way ANOVA with Tukey's multiple comparisons test as post-test, showing statistical difference to solvent control ($^{\bullet}$, P<0.05; $^{\bullet\bullet}$, P<0.01, $^{\bullet\bullet\bullet}$, P<0.001) and E_2 (*, P<0.05; **, P<0.01, ***, P<0.001) was used for statistical analysis. The average ±SEM is of three independent biological experiments done in triplicate. The compounds and extract tested in the presence of E_2 was normalized to E_2, while the compounds and extract tested in the absence of E_2 was normalised to solvent and both are represented by the red dotted line.

Chapter 4

The synergistic combination of SM6Met with 4-OH-Tam (20:1) displays the highest anti-metastatic potential

4.1 Introduction

Breast cancer is a worldwide problem, accounting for 13,7% of cancer deaths and 22,9% of all cancer cases (1), with metastasis to distant organs the main reason for most of these deaths (2). Disrupting metastatic progression is for that reason crucial in reducing breast cancer deaths. It has been argued that all cancers share six distinct hallmarks (Fig. 4.1) that not only direct the transformation of a normal cell into a cancer cell, but also assist in cancer progression and metastasis. These six hallmarks of cancer cells include 1) self-sufficient proliferative signalling (sustaining proliferative signalling); 2) insensitivity to anti-growth signals (evading growth suppressors); 3) ability to evade programed cell death (resisting cell death); 4) infinite replication (enabling replicative immortality); 5) stimulation of blood vessel growth for increased nutrient delivery (inducing angiogenesis) and 6) ability to invade local tissue and disperse to distant tissues (activating invasion and metastasis) (3, 4).

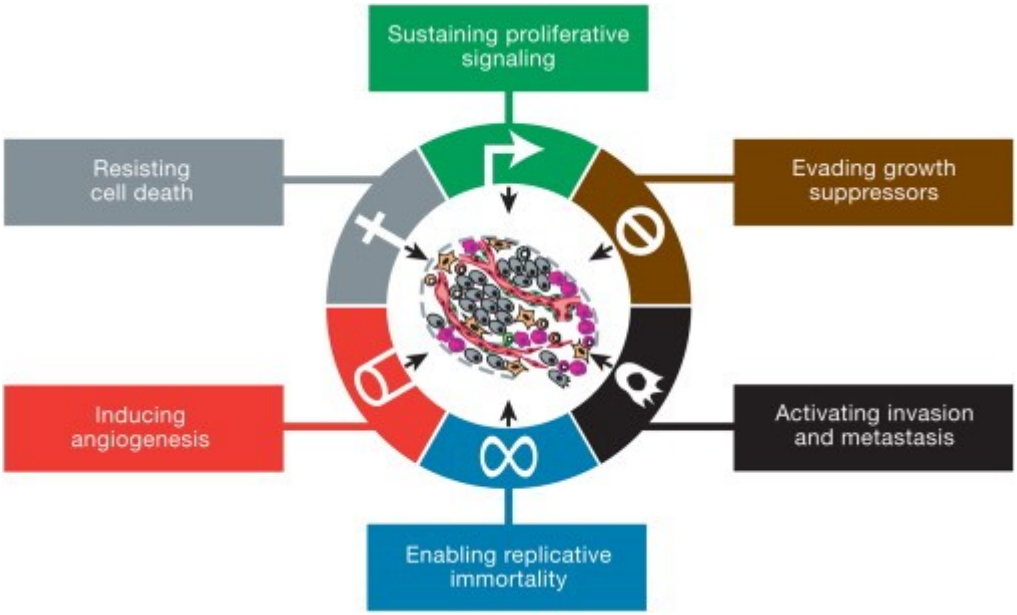

Figure 4.1: The six hallmarks of cancer. Figure taken from Hanahan and Weinberg (4)

It has been argued that the capability of a tumour cell to maintain continuous proliferation is the most fundamental characteristic of tumour cells. Tumour cells sustain continuous proliferation by deregulating the carefully controlled process of cell growth through interfering with the signals involved in regulatory entry into and progression through the cell cycle (3). The cell cycle (Fig. 4.2), of mammalian cells, consists of the G0/G1 phase, the S phase and the G2/M phase (5, 6). Each phase depends on the accurate completion of the phase before it. The cell cycle starts with the G1 phase where the mother cell is checked for DNA damage, which can lead to one out of three possible outcomes. The cell can temporarily enter into the G0 phase (quiescent), the cell can permanently enter into the G0 phase (senescent) or the cell can

continue to synthesize mRNA and proteins in preparation for continuing into the S phase. In the S phase, DNA is replicated and since accurate replication is essential for the survival of the cell, the G1/S transition is an important regulatory checkpoint in the cell cycle. After completion of the S phase the cell enters into the G2 phase, which directly precedes mitosis. In this phase the G2 checkpoint arrests cells with DNA damage and prepares non-damaged cells for mitosis through rapid growth and protein synthesis. Finally the cell enters the M phase (mitosis), where the cell stops growing and undergoes division to generate two identical daughter cells. External and/or intracellular stimuli may lead to a pause in cell cycle progression, often referred to as checkpoints. These checkpoints control cell cycle progression and allow the cell time to repair damaged DNA or to acquire sufficient levels of growth factors required for transition to the next phase. In the event that the DNA damage is too severe at any checkpoint and cannot be repaired, the cell may activate apoptosis.

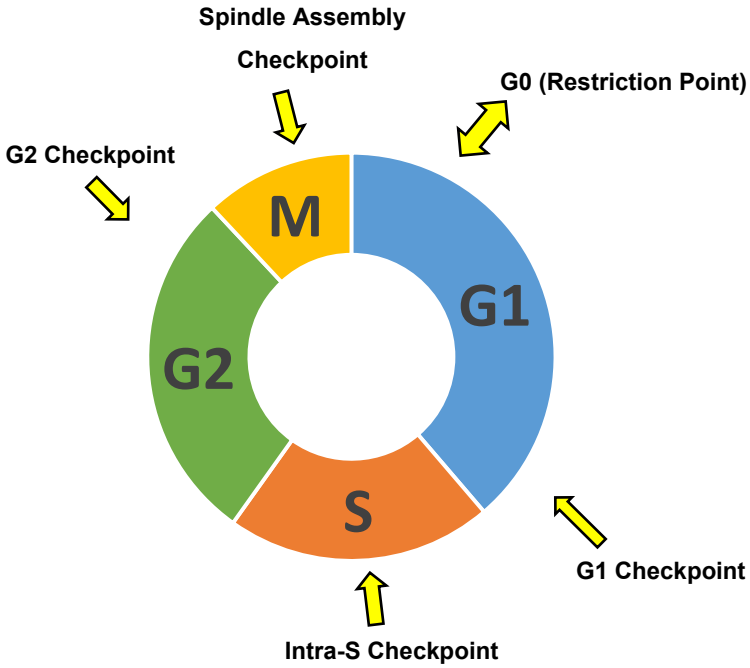

Figure 4.2: Cell cycle phases and regulation by checkpoints. Cell cycle checkpoints regulate the cell cycle and act as barriers to prevent carcinogenesis through maintaining genomic stability. To maintain continuous proliferation tumour cells may deregulate the cell cycle check points (designated by yellow arrows) including: the restriction point (G0), the G1 checkpoint, the intra-S checkpoint, the G2 checkpoint and the mitosis-associated spindle assembly checkpoint. Figure adapted from Bower *et al*. (7)**.**

Once the tumour cell has achieved continuous proliferation, it will produce daughter cells with the same ability to sustain high proliferation. As these daughter cells rapidly proliferate and divide, they acquire more hallmark capabilities that allow these cells to invade and metastasize to other organs (8). Metastasis to distant organs is the leading cause of death amongst breast cancer patients, accounting for about 90% of breast cancer fatalities (9–11). Metastasis is a complex multistep process involving the detachment from the primary tumour, invasion of the basement membrane and entry into the vascular system, survival in circulation, migration to a secondary site and formation of a new colony of tumour cells (secondary tumour) (2, 12). Therefore, cell migration, invasion and colony formation are all potential therapeutic targets to curb metastasis.

Previously (chapter 3) I showed that SM6Met weakly antagonises breast cancer cell proliferation in comparison to SOC therapies like tamoxifen. However, I also showed that the effects of SM6Met on breast cancer cell proliferation could be replicated by combining MPP (ERα antagonist) with liquiritigenin (ERβ agonist) and that SM6Met in combination with 4-OH-Tam synergistically reduced breast cancer cell proliferation. As proliferation is dependent on the cell cycle, I wanted to evaluate the effects of SM6Met on the cell cycle phase distribution of human MCF7-BUS breast cancer cells, in comparison to the rest of the test compounds as well as the previously studied MPP and liquiritigenin combination and SM6Met and 4-OH-Tam combination. In addition, I investigated the effects of the test compounds as well as the two drug combinations (MPP with liquiritigenin and SM6Met with 4-OH-Tam) on the migration, invasion and colony formation (anchorage independent growth) capabilities of the MCF-7BUS cell line, thereby evaluating the effects of the test panel and two drug combinations on the metastatic potential of MCF-7BUS cells.

4.2 Material and Methods

4.2.1 Test compounds used

17β-Estradiol (E_2), a proven agonist of both ER subtypes (13–16); (2)-4-hydroxytamoxifen (the active metabolite of tamoxifen, which will be referred to as 4-OH-Tam in this study), a proven antagonist of both ER subtypes in breast tissue (17–19) and fulvestrant (Ful), a pure antagonist of both ER subtypes in all estrogen target tissues (20–22) were all obtained from Sigma-Aldrich®, South Africa (Sigma). Methyl-piperidino-pyrazole (MPP), a proven ERα selective antagonist (23, 24) and liquiritigenin (Liq), a proven ERβ selective agonist (25) are products of Tocris bioscience, which were obtained from Whitehead Scientific Pty (Ltd), South Africa. The *C. subternata* extract, SM6Met, was previously prepared by a former laboratory member, J.A.K. Visser (16, 26). E_2, 4-OH-Tam, fulvestrant, MPP and Liq stock solutions were prepared in absolute ethanol (EtOH), while SM6Met, stock solutions were prepared in dimethylsulfoxide (DMSO), which was diluted with absolute ethanol to a final concentration of 25%.

4.2.2 Cell culture

The MCF-7BUS human breast cancer cell line (27) (a generous gift from A. Soto, Tufts University, Boston, Massachusetts, United States of America) was maintained in 175cm^2 filter cap culture flasks (SPL Life Sciences) containing, high glucose (4.5g/L) Dulbecco's modified eagle's medium (DMEM) from Sigma, supplemented with heat inactivated 5% (v/v) fetal calf serum (FCS) from Merck, South Africa, 100IU/ml penicillin and 100µl/ml streptomycin (1% Penstrep), 44mM sodium-bicarbonate and 1mM sodium-pyruvate (Sigma). The cells were maintained at 37°C, 97% relative humidity and 5% CO_2 in a humidified cell incubator.

4.2.3 Cell cycle analysis

The human MCF-7BUS breast cancer cells were plated into sterile 10cm^2 tissue culture dishes (Nest Biotechnology Co., Ltd.) at a density of 1 x 10^6 cells/dish and allowed to settle for 24hrs. After settling, the cells were serum starved for four hours by washing the cells once with 10ml sterile, pre-warmed PBS per plate and replacing the medium with unsupplemented DMEM without phenol red (Sigma). Thereafter, the medium was changed to the treatment medium consisting of DMEM without phenol red supplemented with

5% fetal calf serum (Merck), double stripped with dextran coated charcoal and heat inactivated (DS-HI-FCS), and 1% Penstrep and treated with 10^{-11}M E_2, 10^{-9}M 4-OH-Tam, 10^{-9}M fulvestrant, 10^{-6}M MPP, 10^{-9}M liquiritigenin and 0.098µg/ml SM6Met for 48 hours, respectively. After the treatment period the nuclei were isolated and stained using propidium iodide (PI) according to the instructions of the manufacturer of the CycleTEST™ PLUS DNA reagent kit (Bectib Dickinson, South Africa). A 448nm solid state sapphire laser was used to excite the PI stained nuclei and emittance was measured in the PE Texas Red channel on a linear scale using a 616/23 bandpass filter. Histograms were generated of the fluorescent light emitted from the nuclei between 580 and 650nm using the BD FACS Aria Cell sorter from Becton Dickinson manufactured in San Jose, California, USA, and the FACS Diva 6.1.3. software. ModFit LTTM 3.0 software (Verity Software House, Topsham, Maine, USA) was used to analyse the fluorescence histograms to determine cell cycle phase distribution. Final results were presented as fold relative to the average results of the three negative solvent controls including (1) treatment medium, (2) 0.1% (v/v) EtOH in treatment medium and (3) 0.025% (v/v) DMSO in treatment medium.

4.2.4 Scratch-wound healing assay (migration)

MCF-7BUS cells were seeded into 12 well tissue culture plates (Lasec SA [Pty] Ltd) at a density of 1 x 10^6 cells/well and left to incubate until 100% confluency was reached (24-48 hours in general). Once the cells had reached 100% confluency, the medium was changed to phenol red free DMEM supplemented with 5% DS-HI-FCS and 1% Penstrep. Mytomycin C (5µg/ml) was added to each well to inhibit cell proliferation and incubated for two hours. After the incubation, the mixture was aspirated and a “scratch” was made by scraping a vertical wound through the cell monolayer using a sterile 200µl pipette tip after which the cells were washed twice with 400µl sterile, pre-warmed PBS per well to remove any debris. Phenol red free DMEM supplemented with 5% DS-HI-FCS and 1% Penstrep containing the test panel (concentrations indicated in figure legends) was carefully added to each well to avoid detachment of additional cells. The images representing time point zero (T_0) were immediately taken using the Olympus IX81 widefield inverted microscope at the Central Analytical Facility of Stellenbosch University, and thereafter, images where taken at intervals of 24hrs up to time point 72hrs (T_{72}). The images were analysed by measuring the distance between the edges of the wound using ImageJ software (Version 1.49). The distance migrated (moved) was calculated by taking the distance migrated at T_{72} and subtracting it from the distance of the initial wound (T_0) and dividing the answer by the distance of the initial wound (T_0) as depicted in Fig. 4.3.

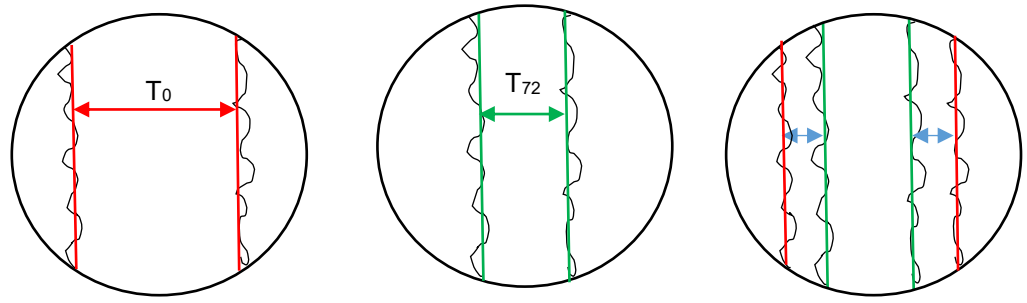

Figure 4.3: Diagram depicting the principle behind the determination of the distance that the cells migrated in the scratch-wound healing assay.

4.2.5. Cell invasion assay

The MCF-7BUS cells were seeded (1x10^6 cells/dish) into 10cm tissue culture dishes and after 24hrs the cells in each plate was washed with 10ml sterile, pre-warmed PBS before the medium was changed to phenol red free DMEM supplemented with 5% DS-HI-FCS and 1% Penstrep and allowed 24hrs to settle. The CytoSelect™ 96-well invasion assay kit (Basement membrane, fluorometric format) from Cell Biolabs, Inc., BIOCOM biotech, South Africa was used to determine the number of invasive cells as described by the manufacturer (Fig. 4.4). In short, the invasion plate consisting of a plate cover (top), membrane chamber insert plate (middle) and feeder tray (bottom), was allowed to warm up to room temperature for 10 minutes after which the basement membrane layers of the membrane chamber insert plate were rehydrated by incubation for 1hr with 100µl of unsupplemented phenol free DMEM in each inner compartment of the 96 well membrane chamber plate. During rehydration the cell suspensions were prepared in treatment medium consisting of unsupplemented phenol free DMEM containing the test compounds or extract (concentrations indicated in figure legends). After rehydration, the medium was removed without disturbing the basement layer and the already prepared cell suspensions were seeded at a density of 5x10^5 cells/chamber into the 96 well membrane chamber plate. The membrane chamber plate was then placed into the feeder tray containing the chemoattractant (DMEM supplemented with 10% DCS-FCS) and left for 24hrs to incubate at 37ºC. After incubation, the membrane chamber plate was removed from the feeder tray containing the chemoattractant and placed into another feeder tray known as the harvesting tray containing the Cell Detachment Solution were the cells that invaded through the membrane were dislodged from the bottom of the membrane. The cells were then lysed and stained with 4x Lysis Buffer/ CyQuant® GR dye (Invitrogen) for 20 minutes at room temperature after which the invasive cells were quantified by measuring fluorescence with a Thermo Scientific™ Varioskan plate reader at 480nm/ 520nm. Final results are presented as fold relative to the average results from the three negative solvent controls including (1) treatment medium, (2) 0.1% (v/v) EtOH in treatment medium and (3) 0.025% (v/v) DMSO in treatment medium.

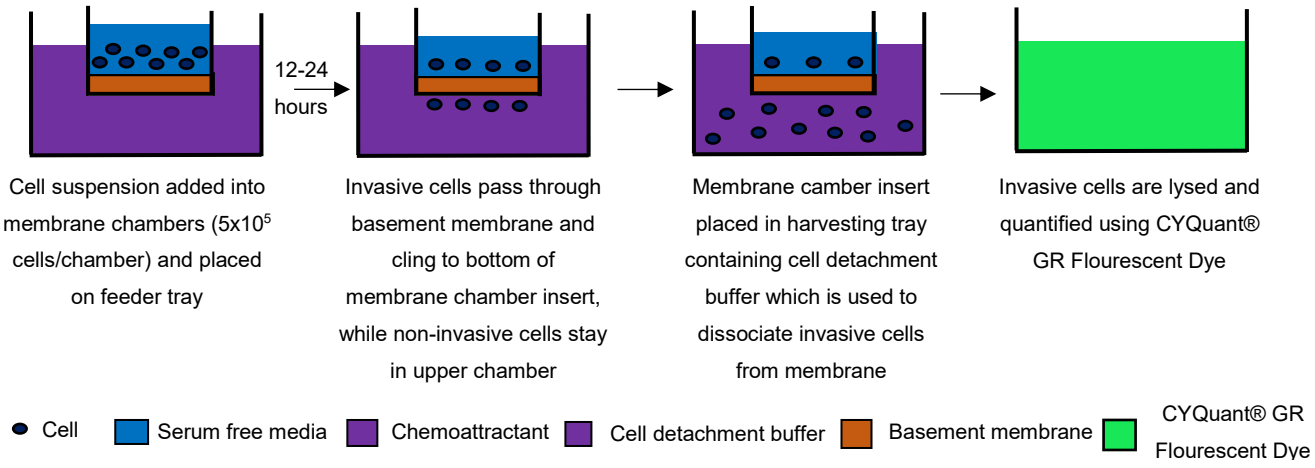

Figure 4.4: Schematic overview of the CytoSelect™ 96-well invasion assay principle

4.2.6 Soft agar colony formation

Soft agar assays were conducted as previously described by Perkins *et al*. (28). In short, MCF-7BUS cells were mixed with phenol red free DMEM supplemented with 5% DS-HI-FCS, 1% Penstrep and 0,6% agarose (Sigma) and plated into a 24 well plate at a density of 1.5 x 10^4 cells/well on top of a solidified layer of phenol red free DMEM supplemented with 5% DS-HI-FCS, 1% Penstrep and 1% agarose. The top cell containing layer was allowed an hour to set at room temperature after which 1ml of treatment medium consisting of phenol red free DMEM supplemented with 5% DS-HI-FCS and 1% Penstrep, containing the test compounds or extract (concentrations indicated in figure legends) was added to the wells (Fig. 4.5). Cells were re-induced and fed weekly for 4 weeks by carefully removing and adding new treatment medium containing the test compounds or extract, without disturbing the cell containing layer. On day 28, the cells were stained overnight with 0.005% crystal violet made up in 10% EtOH (diluted with distilled water, dH_2O). Plates were placed on an illuminated background and photographs were taken, which were analysed using ImageJ software (Version 1.49) to determine the number of colonies formed. Final results are presented as fold relative to the average results from the three negative solvent controls including (1) treatment medium, (2) 0.1% (v/v) EtOH in treatment medium and (3) 0.025% (v/v) DMSO in treatment medium.

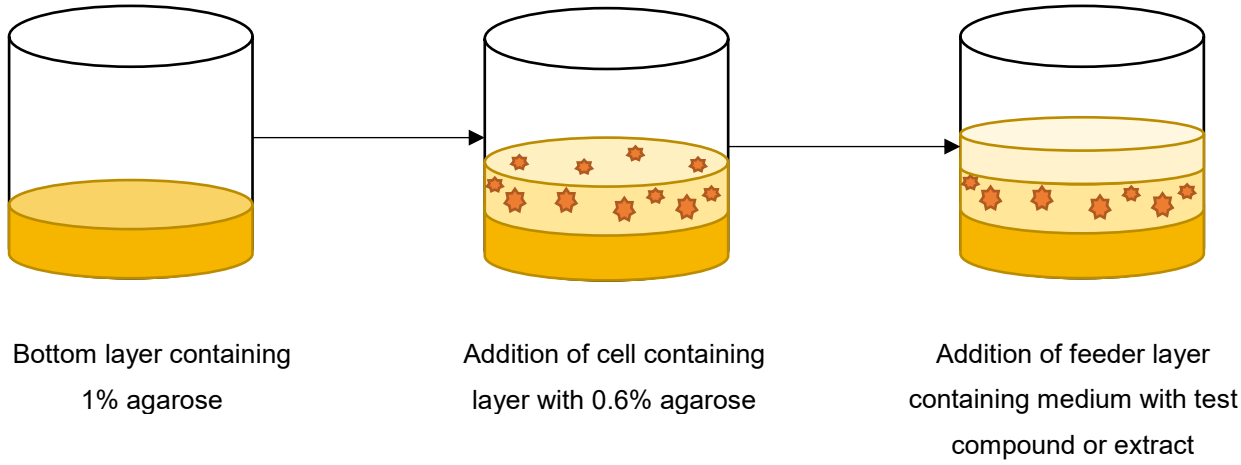

Figure 4.5: Schematic overview of the plating procedure for the soft agar colony formation assay

4.2.7 Statistical analysis of data

GraphPad Prism® version 5.03 for Windows was used for graphical presentation and statistical analysis. One-way ANOVA with Tukey's multiple comparisons test as post-test was used as statistical analysis method (as described in all figure legends). Statistical significance between groups are indicated with different letters and was calculated as a p-value with levels of significance indicated for each experiment.

4.3 Results

4.3.1 Evaluation of the effects of the test compounds and extract on breast cancer cell cycle distribution, in the presence of E_2

The cell cycle is a multifaceted process consisting of four distinct phases i.e. G_1 phase, S phase, G_2 phase and M phase. The accurate progression and completion of each phase is responsible for the activation of every following phase. The cells can also exit the cell cycle and enter a state of temporary quiescence also known as the G_0 phase. The progress of the cell cycle is regulated through checkpoints, which involve a network of regulatory proteins (29). As excessive estrogen signalling is associated with the acquired cancer promoting characteristics described by Hanahan and Weinberg (4), I were interested in investigating the effects of SM6Met in the presence of E_2 on the cell cycle phase distribution.

4.3.1.1 SM6Met induced apoptosis and the accumulation of MCF-7BUS cells in the S phase of the cell cycle.

The effects of the test panel, in the presence of 10^{-11}M E_2, on the distribution of MCF7-BUS breast cancer cells in each phase of the cell cycle as well as the number of apoptotic cells was determined.

The EC_{50} concentration of E_2 as determined from the proliferation studies (Fig. 3.4, Ch. 3) was used for the cell cycle assay, however E_2 showed no significant effect on the cell cycle in comparison to the solvent treated cells (represented by the dashed line, Fig. 4.6B), thereby suggesting, that the EC_{50} concentration of a compound determined by one test system is not always the optimum testing concentration for another test system, as some test systems may be more sensitive. Because E_2 alone at 10^{-11}M E_2 had no significant effect on cell cycle distribution it is difficult to establish whether the effects of the test compounds or extract on the distribution of cells through the cell cycle is a result of the test compounds or extracts on their own or if E_2 contributed to the effects observed (Fig. 4.6, Fig. 4.7 and Fig. 4.8). However, due to time and financial limitations I were not able to redo the cell cycle assay at higher concentrations of E_2.

Nonetheless, with regard to the SOC therapies, relative to solvent 4-OH-Tam showed a slight decrease of cells in the G2/M phase (Fig. 4.6E) with a slight increase of cells in the S phase (Fig. 4.6D) and an 11-fold, although not significant, increase in apoptotic cells (Fig. 4.6F), whereas treatment with fulvestrant significantly ($P<0.05$) increased the number of cells in the G0/G1 phase (Fig. 4.6C), while significantly ($p<0.05$) decreasing cells in the S phase and G2/M phase (Fig. 4.6D & E) relative to solvent and/or E_2. Interestingly, fulvestrant, like E_2, did not cause any cells to go into apoptosis (Fig. 4.6F).

With regard to the two commercially available ER selective ligands, MPP (ERα antagonist) and liquiritigenin (ERβ agonist), significantly ($P<0.05$) decreased the number of cells in the G0/G1 phase (Fig. 4.6C) and significantly ($P<0.01$) increased the number of cells in the S phase (Fig. 4.6D) relative to solvent treated cells. Although MPP and liquiritigenin, increased the number of cells in the apoptotic phase, this was not significant for MPP (Fig. 4.6F).

SM6Met significantly ($P<0.001$) decreased the number of cells in the G0/G1 phase (Fig. 4.6C), while significantly ($P<0.01$) increasing the number of cells in the S phase (Fig. 4.6D) and apoptotic phase (Fig.

4.6F), relative to solvent treated cells. Relative to the other compounds, SM6Met showed the greatest reduction of cells in the G0/G1 phase and the highest accumulation of cells in the S phase.

4.3.1.2 The effects of SM6Met on the cell cycle phases was replicated by combining an ERα selective antagonist (MPP) with an ERβ selective agonist (liquiritigenin)

In the previous chapter, I showed that SM6Met acts as an ERα antagonist and ERβ agonist and not only could I recreate the effects of SM6Met on E_2 induced breast cancer cell proliferation by combining an ERα selective antagonist (MPP) with an ERβ selective agonist (liquiritigenin) (Ch. 3; Fig. 3.2 & 3.3), but this combination also proved to be better at inhibiting E_2 induced breast cancer cell proliferation than the ER subtype selective ligands, MPP and liquiritigenin, alone and the SOC therapies, 4-OH-Tam and fulvestrant. Therefore, I wanted to investigate the combinatorial effects of an ERα selective antagonist (MPP) with an ERβ selective agonist (liquiritigenin) on cell cycle distribution, in the presence of E_2, to determine whether the same trend could be seen for cell cycle distribution.

Combining the IC_{50} concentration of MPP with the IC_{50} concentration of liquiritigenin in a 1:1 ratio resulted in the redistribution of the cells to a cell cycle distribution profile that was not significantly different from that obtained with SM6Met (Fig. 4.7), specifically the 1:1 ratio, like SM6Met alone, decreased the number of cells in the G0/G1 phase (Fig.4.7A), while significantly ($P<0.01$) increasing the number of cells in the S phase (Fig.4.7B) and apoptotic phase (Fig. 4.7D), relative to solvent treated cells.

4.3.1.3 The addition of SM6Met to 4-OH-Tam not only increased the accumulation of cells in the S-phase, but also the number of cells in the apoptotic phase

In Chapter 3, I showed that combining SM6Met with 4-OH-Tam not only resulted in a greater reduction of E_2 induced breast cancer cell proliferation (Ch. 3; Fig. 3.7), but also that SM6Met synergistically enhanced the potency of 4-OH-Tam to reduce breast cancer cell proliferation (Ch. 3; Fig. 3.9 and Fig. 3.10). Therefore, I wanted to evaluate the effect of combining SM6Met with 4-OH-Tam, in ratios (10:1 and 20:1) obtained from Chapter 3, on cell cycle distribution, in the presence of E_2.

The addition of SM6Met to 4-OH-Tam, in the presence of 10^{-11}M E_2, resulted in a dose dependant increase (1.6 fold for the 10:1 ratio and 2.4 fold for the 20:1 ratio) in the number of cells in the S phase and a dose dependant, although not significant, decrease in the number of cells in the G2/M phase (Fig. 4.8B & C). Furthermore, the addition of SM6Met to 4-OH-Tam showed a significant ($P<0.001$) increase (6.9 fold for 10:1 ratio and 7.1 fold for 20:1) in the number of cells in the apoptotic phase in relation to cells treated only with 4-OH-Tam (Fig. 4.8D).

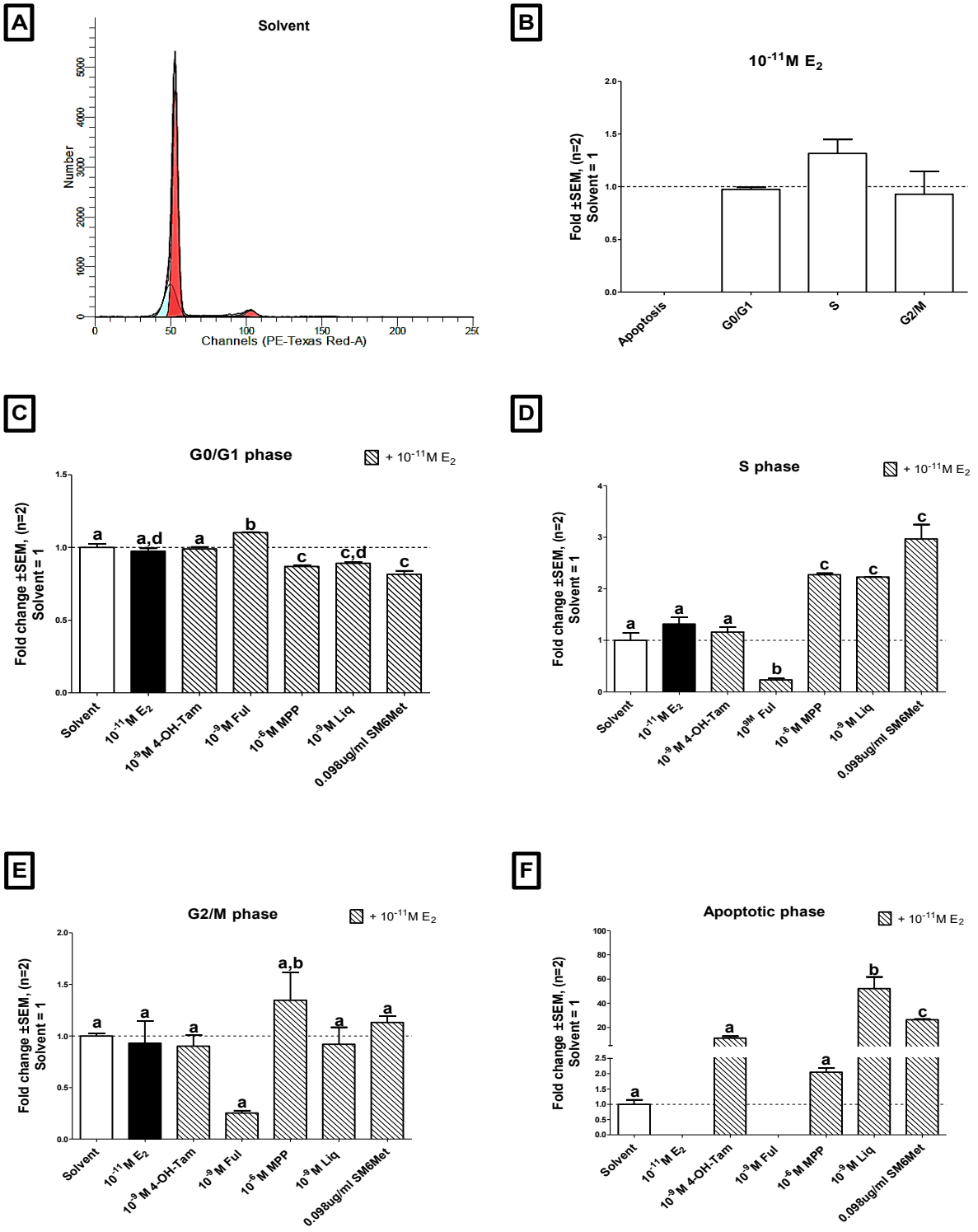

Figure 4.6: Cell cycle analysis of MCF-7BUS cells show that treatment with SM6Met in the presence of 10^{-11}M E_2 induces the accumulation of cells in the S phase. (A), represents the histogram generated by the FACS Diva 6.1.3 software used to analyse the treated cells with solvent. (B), represents the effects of 10^{-11}M E_2 on cell cycle distribution, presented as fold relative solvent. The effects of the IC_{50} concentrations (as determined from Fig. 3.4 in Ch. 3) of 4-OH-Tam, fulvestrant, MPP, liquiritigenin and SM6Met, in the presence of 10^{-11}M E_2 after treatment for 48hrs, on cells in the G_0/G_1 phase (C), S phase (D), G2/M phase (E) and apoptotic phase (F) is presented as fold relative to solvent. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test as post-test, where different letters indicate statistical significance ($P<0.05$). The dotted line through the bars represents the fold induction of the solvent group, which was set to one. Average ± SEM is of two independent biological experiments done in triplicate.

4.3.2 Evaluation of the effects of the test panel on breast cancer metastatic potential

Metastasis is of great importance to cancer research as metastasis is the leading cause of mortality associated all types of cancer (estimated cause of about 90% of all cancer deaths) (30, 31). The process of tumour metastasis, collectively known as the metastatic cascade, involves a chain of events including detachment of cells from the primary tumour, invasion into local tissue, intravasation (migration into the blood stream), survival in circulation, extravasation (exit of tumour cells from circulation) and colonization of tumour cells that leads to the formation of a tumour at a secondary site (2, 12).

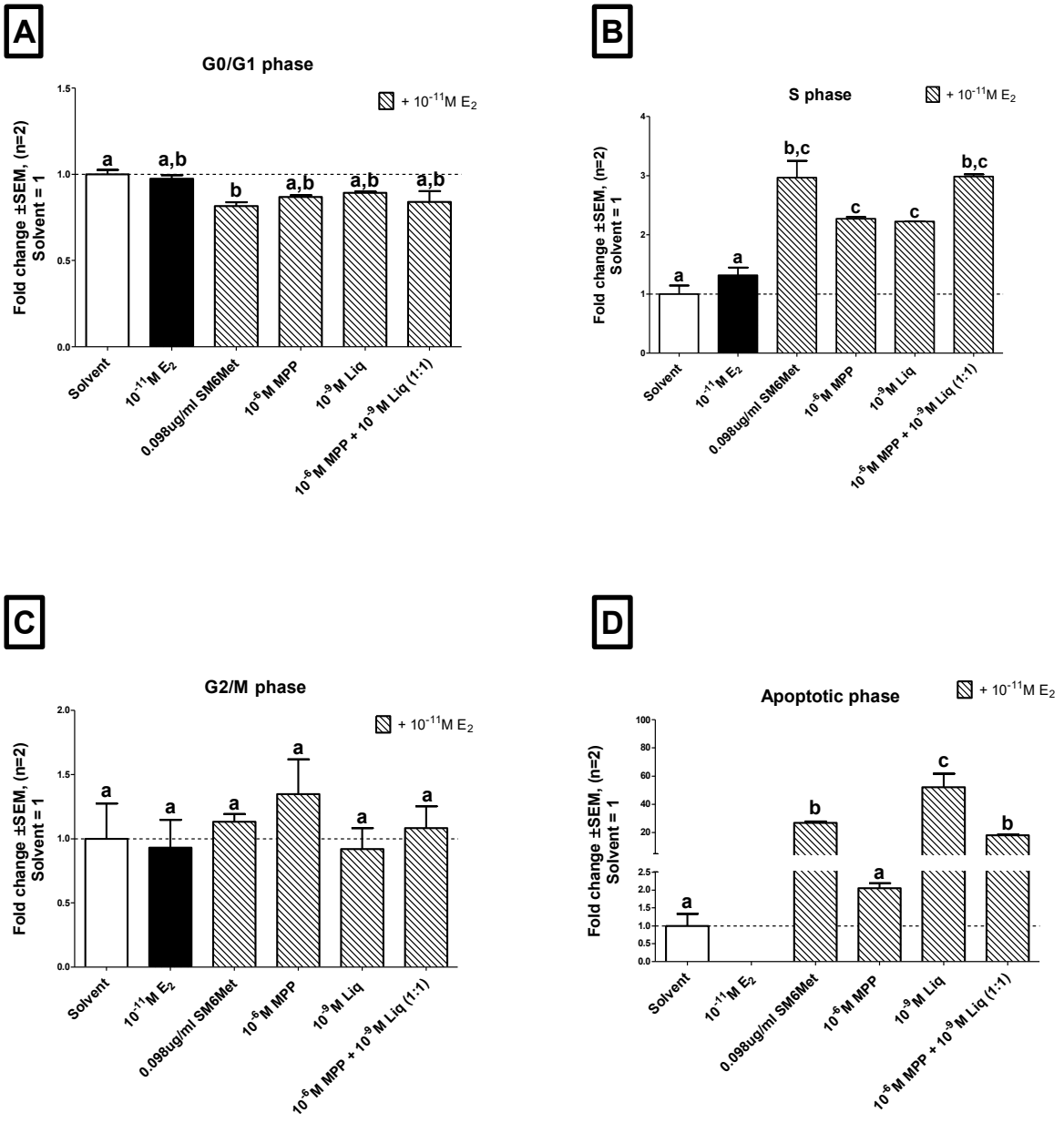

Figure 4.7: Cell cycle analysis of MCF-7BUS cells show that the effect of SM6Met on cell cycle phase distribution can be replicated by combining an ERα selective antagonist with an ERβ selective agonist, in the presence of 10^{-11}M E_2. The effects of the IC_{50} concentrations (as determined from Fig. 3.4 in Ch. 3) of SM6Met, MPP, liquiritigenin and a 1:1 combination of the IC_{50} concentrations of MPP and liquiritigenin after 48hr treatment on cells in the G_0/G_1 phase (A), S phase (B), G2/M phase (C) and apoptotic phase (D) is presented as fold relative to solvent.. Statistical analysis was performed using One-way ANOVA with Tukey's multiple comparisons test as post-test, where different letters indicate statistical significance ($P<0.05$). The dotted line through the bars represents the fold induction

of the solvent group, which was set to one. Average ± SEM is of two independent biological experiments done in triplicate.

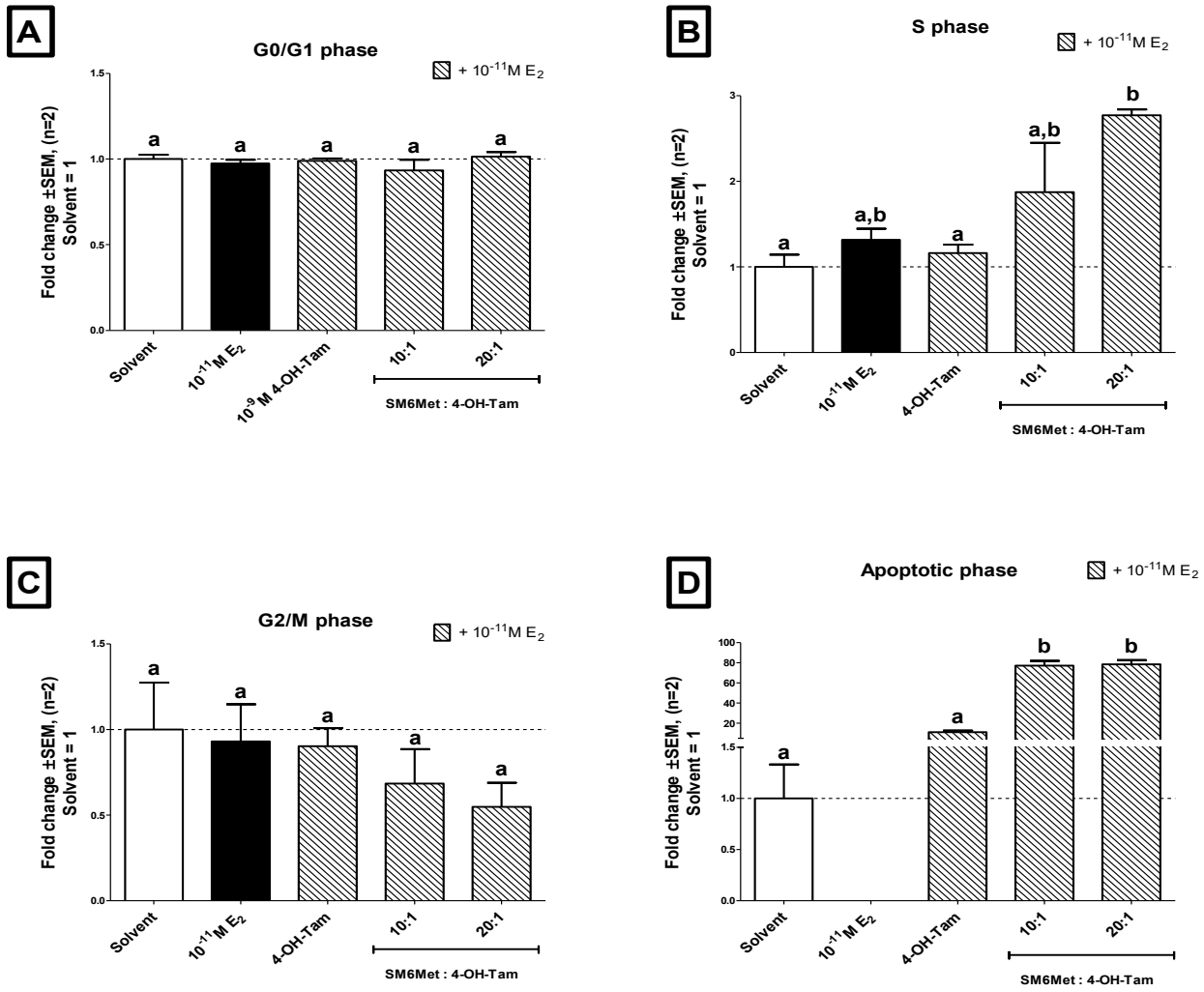

Figure 4.8: Cell cycle analysis of MCF-7BUS cells shows increased accumulation of cells in the S phase and apoptotic phase in response to increasing amounts of SM6Met in the presence of 4-OH-Tam. The effects of the IC_{50} concentrations (as determined from Fig. 3.4 in Ch. 3) of 4-OH-Tam and combinations of SM6Met and 4-OH-Tam in ratios of 10:1 and 20:1 (10 times and 20 times the IC_{50} concentration of SM6Met), in the presence of 10^{-11}M E_2 after 48hr treatment, on cells in the G_0/G_1 phase (A), S phase (B), G2/M phase (C) and apoptotic phase (D) is presented by fold relative to solvent. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test as post-test, where different letters indicate statistical significance ($P<0.05$). The dotted line through the bars represents the solvent control, which was set to one. Average ± SEM is of two independent biological experiments done in triplicate.

These processes like migration, invasion and colony formation could possibly provide new, more selective targets for cancer treatment. Some cancer types have the tendency to metastasise to certain organs, for example breast cancer has a tendency to metastasise to bone (32), lung (33), liver (34) and brain tissue (35). The tendency of certain tumours to metastasise to specific organs was first described by Stephen Paget in 1889 and according to his "seed and soil" theory, cancer cells metastasise to locations with similar microenvironments and characteristics as the tissue they originate from (36–38).

4.3.2.1 Evaluation of the effects of the test panel on breast cancer cell migration

Metastasis can only occur if a cell has the ability to move actively or passively from one location to another. This ability is defined as migration, a process that is dependent on numerous cell activities like the organization of cytoskeletal constituents, extracellular proteolysis and interference with or establishment of the cell matrix and/or cell-cell adhesion (39). The aforementioned cell activities associated with migration, could provide new targets for the development of anti-metastatic therapeutic agents (39). As motility is a prerequisite for invasion and metastasis, I used the *in vitro* scratch assay to evaluate the effects of the test panel on E_2 induced breast cancer cell motility.

4.3.2.1.1 Estradiol reduced breast cancer cell migration, while SM6Met and fulvestrant increased migration to a lesser extent than MPP, the known ERα selective antagonist

The effects of the test panel on migration was evaluated, which involved an incubation period with Mytomycin C to block mitosis (proliferation), thereby, allowing us to differentiate between migration and proliferation to subsequently minimize false results (40, 41). In section 4.3.1, the EC_{50} concentration of E_2 (10^{-11}M, also representative of post-menopausal E_2 levels (42)), was determined from the dose response studies on breast cancer cell proliferation (Fig. 3.4; Ch. 3), but did not have a significant effect on breast cancer cell cycle distribution (Fig. 4.6), thereby suggesting that not all *in vitro* assays are equally sensitive. Therefore, I evaluated breast cancer cell migration not only in the presence of 10^{-11}M E_2, but also at 10^{-9}M E_2, another physiologically relevant concentration as it reflects the pre-menopausal levels of E_2 in the human body (42).

Although 10^{-11}M E_2 decreased the migration of the human MCF-7BUS cells slightly, it did not reach a level of significance, while induction with 10^{-9}M E_2 significantly ($P<0.01$) decreased the migration of these cells (Fig. 4.9). In addition, none of the test compounds or the extract, in the presence of 10^{-11}M E_2, had a significant effect on breast cancer cell migration (Fig. 4.9B). However, in the presence of 10^{-9}M E_2, all the test compounds and the extract counteracted the effects of 10^{-9}M E_2, by significantly increasing cell motility in comparison to cells treated with 10^{-9}M E_2.

Specifically, the two SOC therapies, 4-OH-Tam and fulvestrant induced similar levels of migration, in the presence of 10^{-9}M E_2. Interestingly, although liquiritigenin (ERβ selective agonist) displayed slightly higher induction of breast cancer cell migration in relation to 4-OH-Tam and fulvestrant, the difference was not statistically significant. MPP, the ERα selective antagonist, displayed the highest induction of breast cancer cell migration, whereas SM6Met displayed the lowest induction of breast cancer cell migration among the compounds tested. Collectively, the results illustrate that all the test compounds as well as the extract, SM6Met, counteracted (to varying degrees) the protective effects of E_2 on the migration of the human MCF-7BUS breast cancer cells *in vitro*.

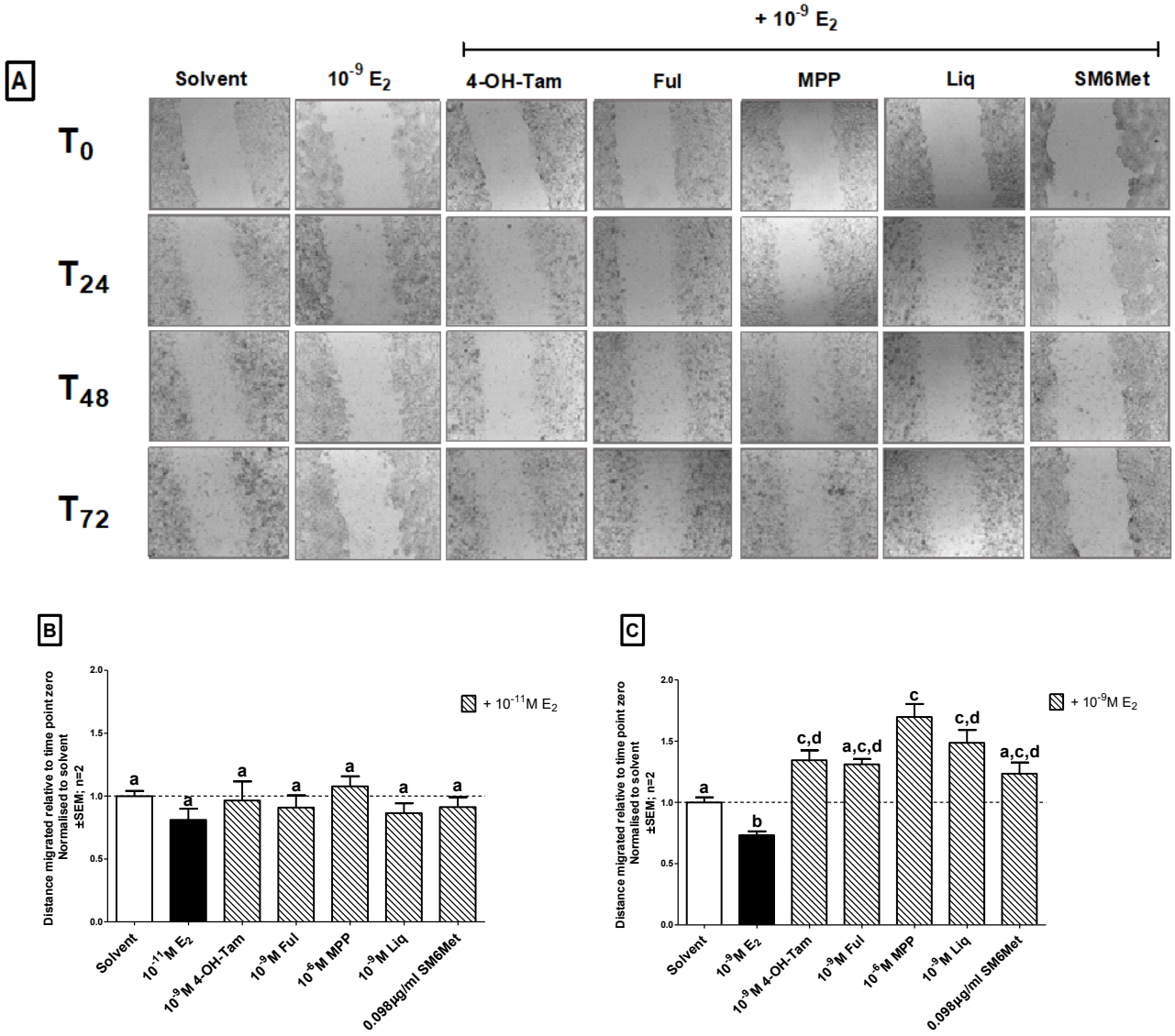

Figure 4.9: Estradiol reduced breast cancer cell migration, while SM6Met and fulvestrant increased migration to a lesser extent than the ERα selective antagonist, MPP. Immediately after induction with the test panel (4-OH-Tam, fulvestrant, MPP, liquiritigenin and SM6Met, all in the presence of either 10^{-11}M or 10^{-9}M E_2) an image was captured using an Olympus IX81 widefield microscope at 10x magnification and will be referred to as time point zero (T_0). (A), represents the wound at 24hr intervals, starting at time point zero and ending at 72hrs. The distance migrated was calculated using the formula T_{72}-T_0/T_0 for each compound or extract and normalised to solvent (B and C). Statistical analysis was performed using One-way ANOVA with Tukey's multiple comparisons test as post-test, where different letters indicate statistical significance ($P<0.05$). The dotted line through the bars represents the solvent, which was set to one. Average ± SEM is of two independent biological experiments done in triplicate.

4.3.2.1.2 The increased migratory effect of MPP (ERα selective antagonist) was reduced to the same level as the solvent control when combined with liquiritigenin (ERβ selective agonist)

Liquiritigenin was combined with MPP to mimic the ER subtype characteristics of SM6Met and to evaluate whether this combination would deliver the same effects as SM6Met on breast cancer cell migration. For this reason, the IC_{50} concentrations (as determined from proliferation studies, Fig. 3.4 of Ch. 3) of liquiritigenin (ERβ selective agonist) and MPP (ERα selective antagonist) were used in combination ratios of 1:1, 10:1, 100:1 and 1000:1. Increasing the concentration of liquiritigenin in the combination resulted in

the reduction of the high migratory effect of MPP, in a dose dependant manner, to the same level as the solvent treated cells.

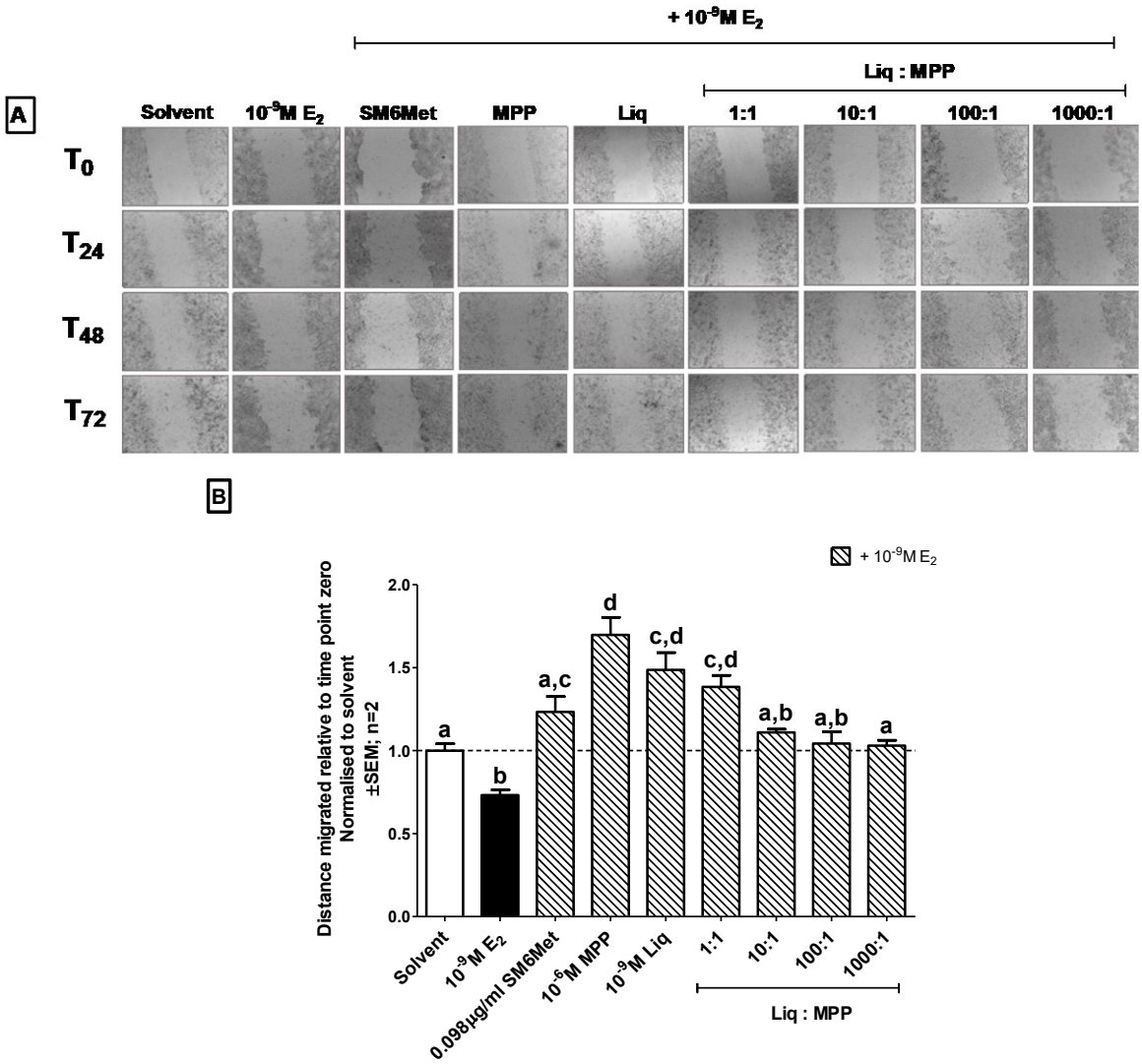

Figure 4.10: Combining increasing concentrations of liquiritigenin with MPP reduced the migratory effect of MPP on human MCF-7BUS breast cancer cells to the same level as the solvent control. Immediately after induction with the test panel (SM6Met, MPP, liquiritigenin and MPP in combination with increasing concentrations of liquiritigenin, all in the presence of 10^{-9}M E_2) an image was captured using an Olympus IX81 widefield microscope at 10x magnification and will be referred to as time point zero (T_0). (A), represents the wound at 24hr intervals, starting at time point zero and ending at 72hrs. The distance migrated was calculated using the formula T_{72}-T_0/T_0 for each compound or extract and normalised to solvent (B). Statistical analysis was performed using One-way ANOVA with Tukey's multiple comparisons test as post-test, where different letters indicate statistical significance ($P<0.05$). The dotted line through the bars represents the solvent, which was set to one. Average ± SEM is of two independent biological experiments done in triplicate.

The results suggest that a lower concentration of the ERα antagonist and a higher concentration of the ERβ agonist could be more advantageous for the reduction of breast cancer cell migration. In summary, I showed that the effects of SM6Met on cell migration could be replicated by the addition of an ERβ agonist (liquiritigenin) to an ERα antagonist (MPP) (Fig. 4.10B) and that increasing concentrations of the ERβ agonist in the combination could potentially reduce breast cancer cell migration even further.

4.3.2.1.3 Combining SM6Met with tamoxifen in a ratio of 20:1 completely reversed the migratory potential, leading to an overall reduction in migration

In support of the role ERβ plays in the regulation and inhibition of breast cancer cell migration (43, 44), increasing the concentration of SM6Met (increasing ERβ activity) in combination with 4-OH-Tam resulted in dose dependant significant breast cancer cell migration inhibition (Fig. 4.11). At a ratio of 20:1 this reduction was not significantly different from the level of inhibition produced by 10^{-9}M E_2 on its own.

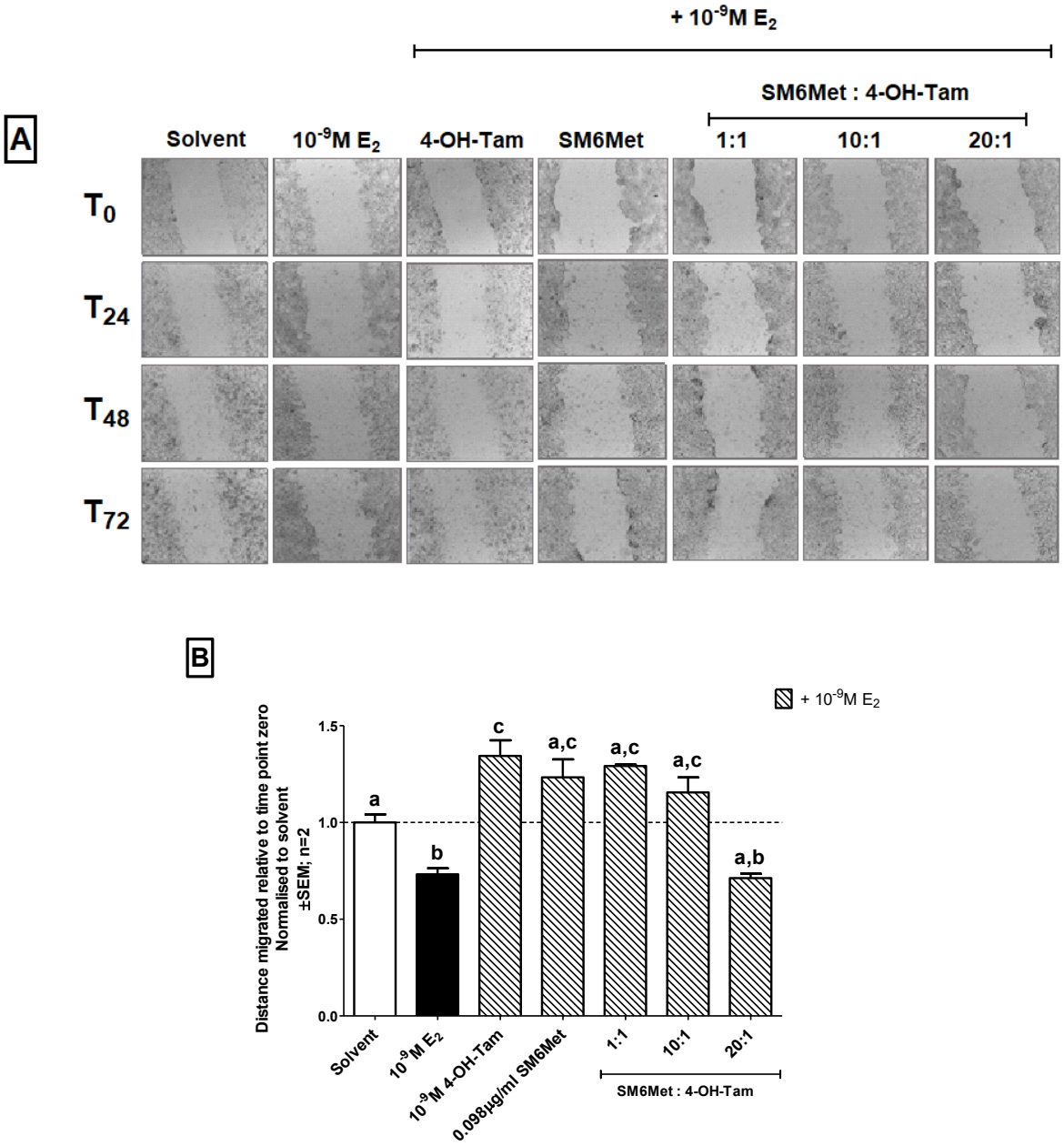

Figure 4.11: Increasing the concentration of SM6Met in the ratio to which it is combined with 4-OH-Tam reduced breast cancer cell migration up to the same extent as E_2. Immediately after induction with the test panel (SM6Met, 4-OH-Tam, SM6Met and 4-OH-Tam in combination with increasing concentrations of SM6Met, all in the presence of 10^{-9}M E_2) an image was captured using an Olympus IX81 widefield microscope at 10x magnification and will be referred to as time point zero (T_0). (A), represents the wound at 24hr intervals, starting at time point zero and ending at 72hrs. The distance migrated was calculated using the formula T_{72}-T_0/T_0 for each compound or extract and normalised to solvent (B). Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test as post-test, where different letters indicate statistical significance ($P<0.05$). The dotted line through the bars represents the solvent, which was set to one. Average ± SEM is of two independent biological experiments done in triplicate.

4.3.2.2 Evaluation of the effects of the test panel on breast cancer cell invasion

Cell invasion is closely related to cell migration and is defined as the ability to penetrate neighbouring tissues via the lymphatic and blood vessels by navigating through the extracellular matrix (ECM) to enter into the circulation and invade tissue elsewhere in the body to form secondary tumours (4, 45). As cell penetration through the basal membrane is a prerequisite for cell invasion and metastasis, the effects of the test panel on breast cancer cell invasion was investigated.

4.3.2.2.1 SM6Met, decreased the number of invasive MCF-7BUS cells in the absence and presence of E_2

All the test compounds and the extract, on their own, significantly ($P<0.001$) decreased the number of invasive MCF-7BUS cells (Fig. 4.12A). In addition, SM6Met displayed significantly higher reduction of invasive MCF-7BUS cells than the SOC therapies on their own (4-OH-Tam and fulvestrant) or the ERα selective antagonist (MPP) on its own. No significant change in the number of invasive cells was observed after treatment with 10^{-11}M E_2 on its own, while treatment with 10^{-9}M E_2 resulted in a significant ($P<0.001$) increase in the number of invasive MCF-7BUS cells in relation to solvent treated cells.

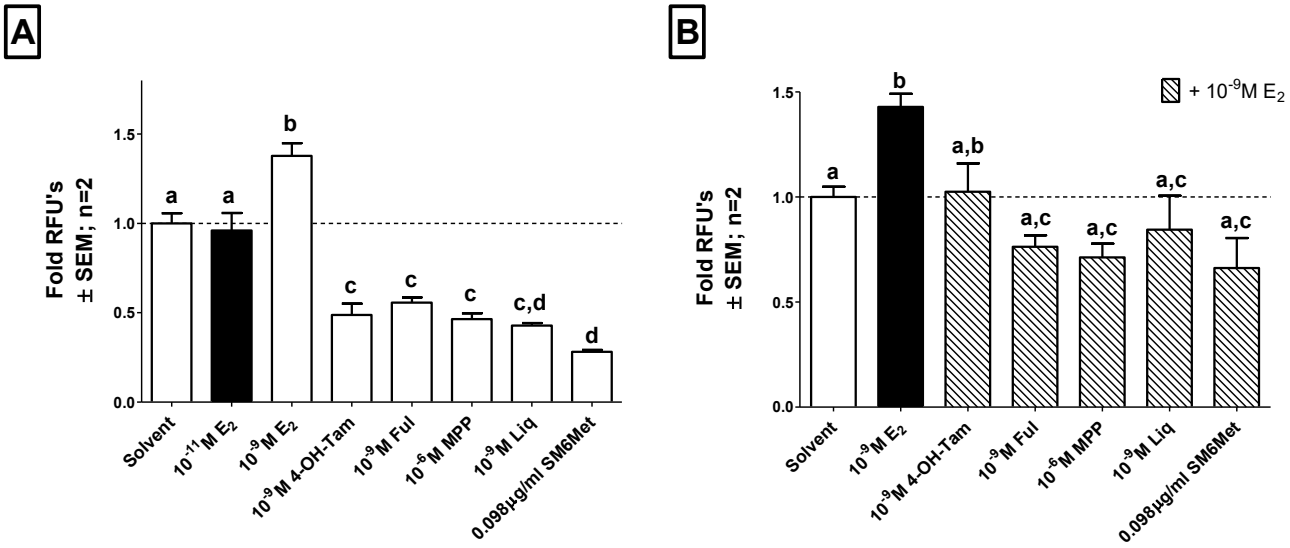

Figure 4.12: SM6Met, decreased the number of invasive MCF-7BUS cells in the absence and presence of E_2. The number of invasive MCF-7BUS cells was determined using the CytoSelect™ 96-Well cell invasion assay kit as described in the material and methods section. (A) displays the effects of the test panel (4-OH-Tam, fulvestrant, MPP, liquiritigenin and SM6Met) on the number of invasive cells in the absence of E_2, while (B) demonstrate the effect of the test panel on the number of invasive cells in the presence of 10^{-9}M E_2. Statistical analysis was performed using One-way ANOVA with Tukey's multiple comparisons test as post-test, where different letters indicate statistical significance ($P<0.05$). The dotted line through the bars represents the solvent, which was set to one. Average ± SEM is of two independent biological experiments done in triplicate.

In the presence of 10^{-9}M E_2, the test panel no longer significantly reduced MCF-7BUS cell invasion in relation to solvent treated cells, however the whole test panel, with the exception of 4-OH-Tam, significantly decreased E_2 induced invasion (Fig. 4.12B). Despite slight differences between the levels of inhibition elicited by the test compounds and extract, no statistical difference was found between the effects. In

summary, it appears that in the presence of 10^{-9}M E_2 the test panel is less effective at inhibiting cell invasion relative to solvent, but is still able to antagonise, with the exception of 4-OH-Tam, the effects of E_2 alone.

4.3.2.2.2 Combining MPP with liquiritigenin slightly reduced the number of invasive cells

In Fig. 4.13A, liquiritigenin and MPP significantly (P<0.001) inhibited MCF-7BUS cell invasion on their own. However, treatment with SM6Met on its own resulted in an inhibition of MCF-7BUS cell invasion which was significantly higher than the effect of liquiritigenin (P<0.05) or MPP (P<0.01). By combining MPP and liquiritigenin in a 1:1 ratio (10^{-6}M MPP + 10^{-9}M Liq) the inhibitory effect of the two compounds, although not significantly different to the compounds alone, was slightly enhanced. Furthermore, the 1:1 ratio was not significantly different from SM6Met alone, suggesting that the effects of SM6Met on breast cancer cell invasion could be replicated by combining two compounds that represent the subtype specific characteristics of SM6Met i.e. MPP (ERα antagonist) and liquiritigenin (ERβ agonist).

Once again, with the addition of 10^{-9}M E_2 the test panel (SM6Met, MPP, liquiritigenin and the 1:1 combination of MPP with liquiritigenin) became less effective at inhibiting cell invasion relative to solvent, but was still able to significantly antagonise the effect of 10^{-9}M E_2 (Fig. 4.13B). In the presence of 10^{-9}M E_2, no significant difference was found between MPP, liquiritigenin, the 1:1 ratio (10^{-6}M MPP + 10^{-9}M Liq) and SM6Met. However, the inhibitory effects of the MPP and liquiritigenin alone, although not significantly so, were slightly enhanced through the combination of the two compounds.

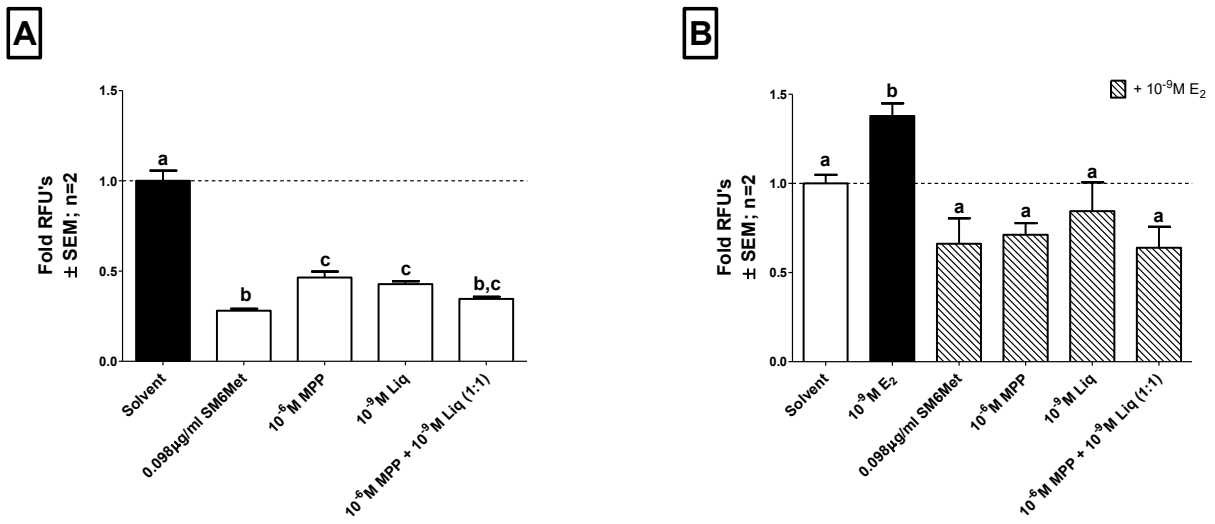

Figure 4.13: Combining MPP with liquiritigenin resulted in a slight decrease in the number of invasive MCF7-BUS cells, almost to the same extent as SM6Met. The number of invasive MCF-7BUS cells was determined using the CytoSelect™ 96-Well cell invasion assay kit as described in the material and methods section. (A) displays the effects of the test panel (SM6Met, MPP, liquiritigenin and a 1:1 combination of the IC_{50} concentrations of MPP with liquiritigenin) on the number of invasive MCF-7BUS cells in the absence of E_2, while (B) shows the effects of the test panel on the number of invasive MCF-7BUS cells in the presence of 10^{-9}M E_2. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test as post-test, where different letters indicate statistical significance (P<0.05). The dotted line through the bars represents the solvent, which was set to one. Average ± SEM is of two independent biological experiments done in triplicate.

4.3.2.2.3 SM6Met in combination with 4-OH-Tam, in the presence of 10^{-9}M E_2, reduced the number of invasive cells even more than 4-OH-Tam on its own

In Fig. 4.14A, SM6Met on its own could reduce MCF-7BUS cell invasion to a significantly (P<0.01) greater extent than 4-OH-Tam alone. However, the results suggest that in combination 4-OH-Tam initially outcompetes the effect of SM6Met. Nonetheless, increasing the concentration of SM6Met in the combination ratio with 4-OH-Tam resulted in a slight increase of the inhibitory effects relative to 4-OH-Tam alone.

In the presence of 10^{-9}M E_2 (Fig. 4.14B), 4-OH-Tam was able to significantly reduce E_2 induced cell invasion. SM6Met alone, also, significantly (P<0.001) reduced E_2 induced cell invasion. The addition of SM6Met to 4-OH-Tam resulted in significant (P<0.05) reduction of cell invasion relative 10^{-9}M E_2 as well as to solvent, with the highest inhibition elicited by the 20:1 combination ratio of SM6Met with 4-OH-Tam. Furthermore, the combination of SM6Met with 4-OH-Tam shows significantly (P<0.05) higher inhibition of E_2 induced breast cancer cell invasion than either 4-OH-Tam or SM6Met alone.

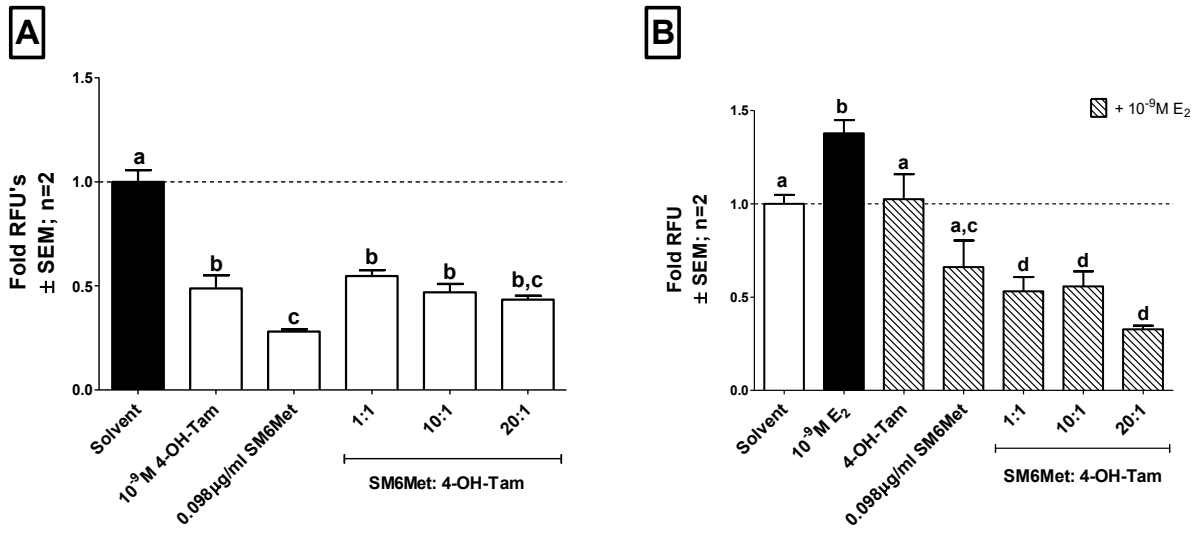

Figure 4.14: SM6Met in combination with 4-OH-Tam, in the presence of 10^{-9}M E_2, is more effective than 4-OH-Tam alone at reducing the number of invasive MCF-7BUS cells. The number of invasive MCF-7BUS cells was determined using the CytoSelect™ 96-Well cell invasion assay kit as described in the material and methods section. (A) displays the effects of the test panel (4-OH-Tam, SM6Met and the IC_{50} ratio combinations 1:1, 10:1 and 20:1 of 4-OH-Tam with SM6Met) on the number of invasive cells in the absence of E_2, while (B) shows the effects of the test panel on the number of invasive cells in the presence of 10^{-9}M E_2. Statistical analysis was performed using One-way ANOVA with Tukey's multiple comparisons test as post-test, where different letters indicate statistical significance (P<0.05). The dotted line through the bars represents the solvent, which was set to one. Average ± SEM is of two independent biological experiments done in triplicate.

4.3.2.3 Evaluation of the effects of the test panel on breast cancer colony formation

In the event that a cell detaches from the ECM, the cell will undergo apoptosis also known as detachment-apoptosis or anoikis (45). Anoikis is an important physiological defence system that prevents cell re-attachment at incorrect locations. The ability of tumour cells to evade anoikis, grow independently and form a new colony is known as anchorage-independent growth (4, 45). Anchorage independent growth is not only a hallmark of cancer, but is also a characteristic needed for cancer metastasis. Therefore, I evaluated

the effects of the test panel on anchorage-independent growth of the MCF-7BUS cell line using the soft-agar colony formation assay (28, 46).

4.3.2.3.1 SM6Met reduced MCF-7BUS cell colony formation to the same extent as 4-OH-Tam and liquiritigenin in the presence of E_2

E_2 was able to inhibit the formation of colonies in a dose-dependent manner from 10^{-9}M E_2 (Fig. 4.15), and consequently at high concentrations of E_2 the effects of the test panel was over shadowed by the effect of E_2. Thus, discrimination between the effects of test panel was evaluated at lower concentrations of E_2.

In the presence of 10^{-11}M E_2 (Fig. 4.15A & B), 4-OH-Tam, liquiritigenin and SM6Met displayed similar inhibition levels. Fulvestrant and MPP were both able to inhibit colony formation more effectively ($P<0.01$) than SM6Met, with MPP displaying the highest level of inhibition.

In the presence of 10^{-9}M E_2 (Fig. 4.15C), 4-OH-Tam and MPP were the only compounds to significantly ($P<0.05$) reduce colony formation relative to 10^{-9}M E_2 on its own. The overpowering effect of E_2, made comparing the test compounds to SM6Met increasingly difficult as no statistical difference was observed between the inhibition levels of the test compounds and the extract in the presence of 10^{-7}M and 10^{-5}M E_2 (Fig. 4.13D & E).

4.3.2.3.2 MPP displayed a strong inhibiting effect on colony formation, in the presence of E_2, thereby in combination overshadowing the effects of liquiritigenin.

In the presence of 10^{-11}M E_2 (Fig. 4.16), SM6Met, MPP, liquiritigenin and the 1:1 combination of liquiritigenin with MPP were all able to significantly reduce colony formation relative to solvent and 10^{-11}M E_2. However, MPP displayed a significantly ($P<0.001$) 8.5-fold greater inhibition than SM6Met and a 10-fold greater inhibition than liquiritigenin. Although the addition of liquiritigenin to MPP resulted in a slight 2.96-fold increase in the inhibitory effect of MPP, it was not statistically different to that of MPP on its own in the presence of 10^{-11}M E_2. The combination of liquiritigenin and MPP, like MPP alone, showed significantly higher inhibition of colony formation than SM6Met alone. The over shadowing effects of 10^{-6}M MPP suggests that ERα plays a pivotal role in colony formation.

4.3.2.3.3 All the combinations of SM6Met with 4-OH-Tam showed greater reduction in colony formation in comparison to SM6Met and 4-OH-Tam on their own

In the presence of 10^{-11}M E_2 (Fig. 4.17), SM6Met displayed a similar level of inhibition of colony formation as 4-OH-Tam, however, when SM6Met was combined with 4-OH-Tam in a 1:1 ratio it resulted in significantly ($P<0.001$) 10-fold further reduction of colony formation. The level of inhibition, however, did not significantly change when the concentration of SM6Met was increased in the combination ratio with 4-OH-Tam suggesting that the 1:1 combination ratio is efficient in reducing colony formation and that higher concentrations of SM6Met are not required.

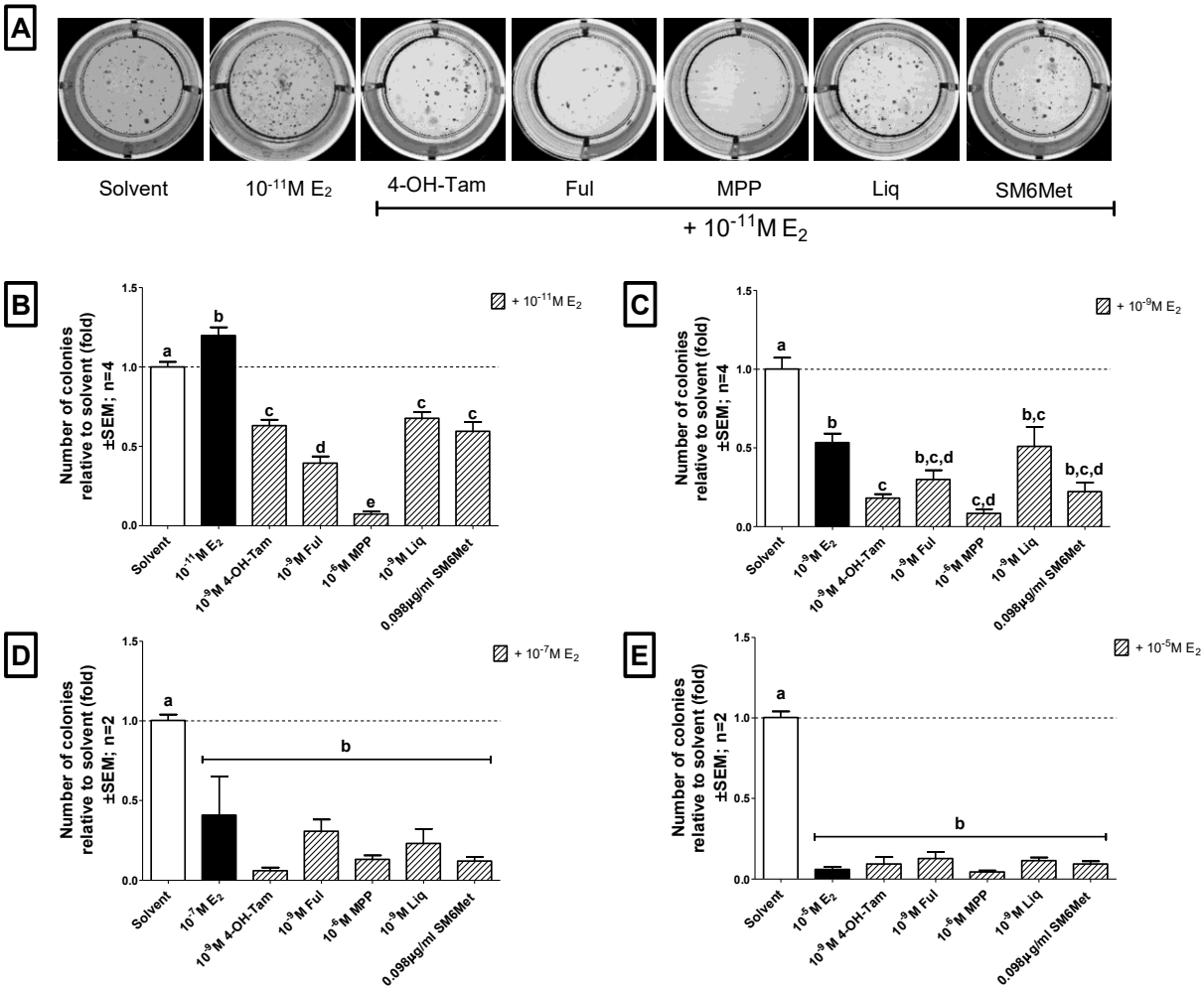

Figure 4.15: SM6Met reduced MCF-7BUS cell colony formation to the same extent as 4-OH-Tam and liquiritigenin in the presence of E_2. MCF7-BUS cells were suspended in 0.6% agarose and added onto the bottom 1% agarose layer. The cells were treated weekly with the test panel including 4-OH-Tam, fulvestrant, MPP, liquiritigenin and SM6Met, all in the presence of (B) 10^{-11}M E_2, (C) 10^{-9}M E_2, (D) 10^{-7}M E_2 and (E) 10^{-5}M E_2 for the duration of 21 days. At day 21, images were taken and the number of colonies formed were counted using ImageJ software. Representative images of the test panel in the presence of 10^{-11}M E_2 is shown (A). Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test as post-test, where different letters indicate statistical significance ($P<0.05$). The dotted line through the bars represents the solvent, which was set to one. Average ± SEM is of two independent biological experiments done in triplicate.

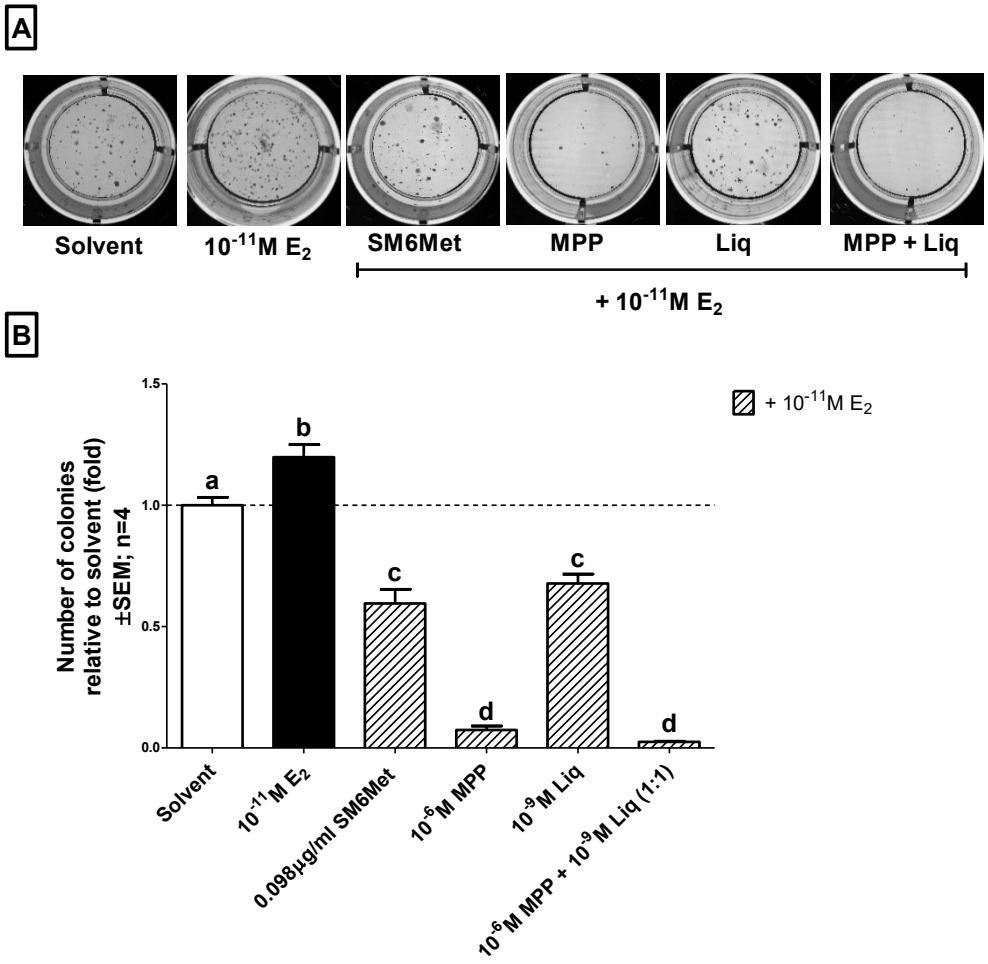

Figure 4.16: MPP displayed a strong inhibiting effect on colony formation, in the presence of E_2, thereby, in combination, overshadowing the effects of liquiritigenin. MCF7-BUS cells were suspended in 0.6% agarose and added onto the bottom 1% agarose layer. The cells were treated weekly with the test panel including SM6Met, MPP, liquiritigenin and the IC_{50} concentrations of MPP and liquiritigenin in a 1:1 ratio, all in the presence of 10^{-11}M E_2 for the duration of 21 days (B). At day 21, images were taken and the number of colonies formed were counted using ImageJ software. Representative images of compound or extract in the presence of 10^{-11}M E_2 is shown (A). Statistical analysis was performed using One-way ANOVA with Tukey's multiple comparisons test as post-test, where different letters indicate statistical significance ($P<0.05$). The dotted line through the bars represents the solvent, which was set to one. Average ± SEM is of two independent biological experiments done in triplicate.

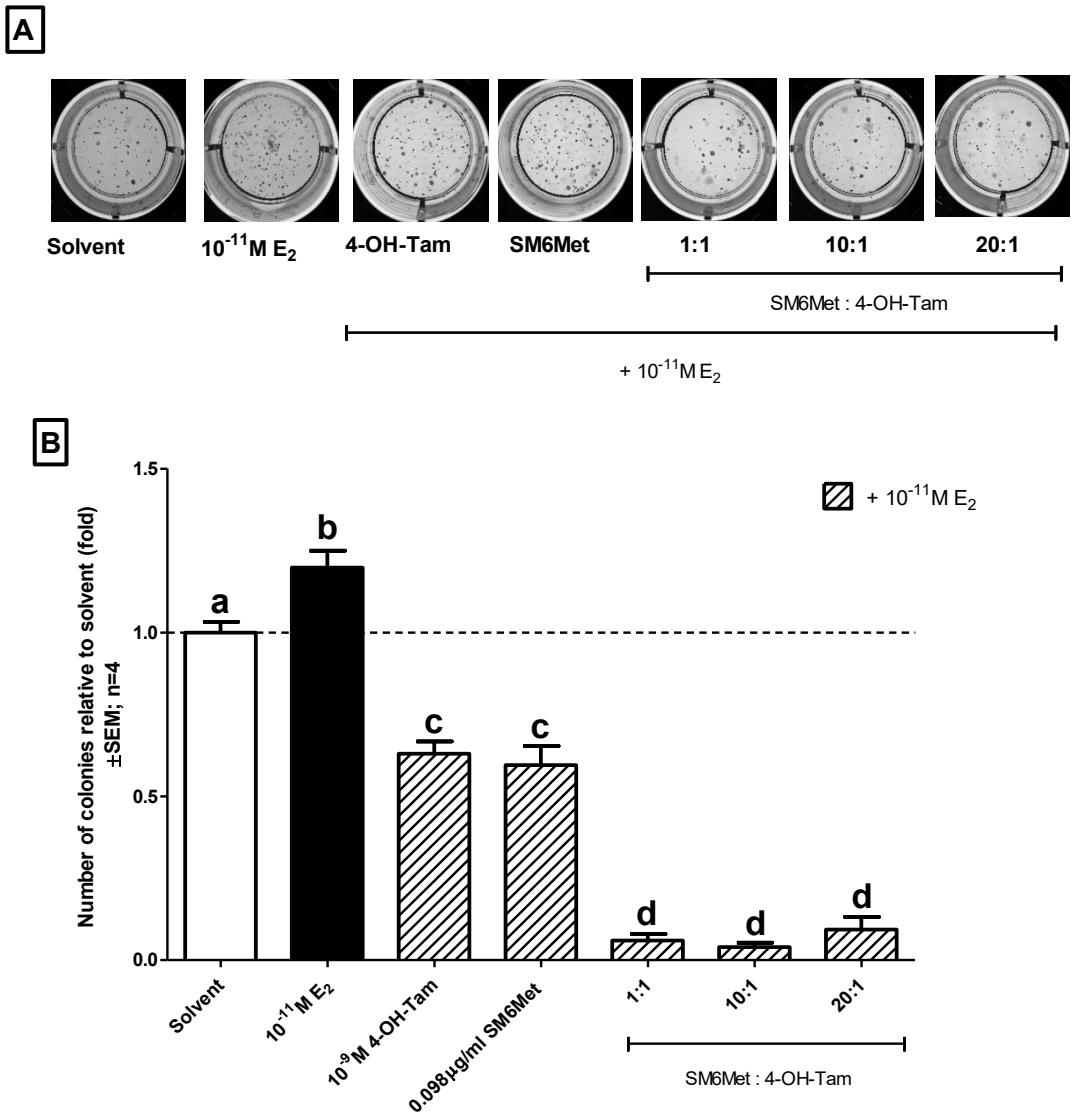

Figure 4.17: All the combinations of SM6Met with 4-OH-Tam showed greater inhibition of colony formation in comparison to SM6Met and 4-OH-Tam on their own. MCF7-BUS cells were suspended in 0.6% agarose and added onto the bottom 1% agarose layer. The cells were treated weekly with the test panel including 4-OH-Tam, SM6Met, and 4-OH-Tam in combinations with SM6Met at 1:1, 10:1 and 20:1 ratios, all in the presence of 10^{-11}M E_2 for the duration of 21 days (B). At day 21, images were taken and the number of colonies formed were counted using ImageJ software. Representative images of compound or extract in the presence of 10^{-11}M E_2 is shown (A). Statistical analysis was performed using One-way ANOVA with Tukey's multiple comparisons test as post-test, where different letters indicate statistical significance ($P<0.05$). The dotted line through the bars represents the solvent, which was set to one. Average ± SEM is of two independent biological experiments done in triplicate.

Table 7: Summary of the effects of the test panel on the metastatic potential of the human MCF-7BUS breast cancer cells

		Cell cycle assay[a]				Migration assay[b]	Invasion assay[b]	Colony formation assay[a]
		G0/G1 phase	S phase	G2/M phase	Apoptosis			
10^{-11} M E_2		-	-	-	-	-	-	$\uparrow^{\bullet\bullet\bullet}$
10^{-9} M E_2		nd	nd	nd	nd	$\downarrow^{\bullet\bullet}$	$\uparrow^{\bullet\bullet\bullet}$	$\downarrow^{\bullet\bullet\bullet}$
Ful		$\uparrow^{*}$	$\downarrow^{**}$	$\downarrow^{*}$	-	$\uparrow^{***}$	$\downarrow^{***}$	$\downarrow^{***}$
4-OH-Tam		-	-	-	-	$\uparrow^{***}$	-	$\downarrow^{***}$
SM6Met		$\downarrow^{**}$	$\uparrow^{***}$	-	$\uparrow^{**}$	$\uparrow^{**}$	$\downarrow^{***}$	$\downarrow^{***}$
SM6Met:4-OH-Tam[c/d,e]	**1:1**	nd	nd	nd	nd	$\uparrow^{***,ns}$	$\downarrow^{***,\$\$}$	$\downarrow^{***,\$\$\$}$
	10:1	-	-	-	$\uparrow^{***,\$\$\$}$	$\uparrow^{**,ns}$	$\downarrow^{***,\$\$}$	$\downarrow^{***,\$\$\$}$
	20:1	-	$\uparrow^{*,\$}$	-	$\uparrow^{***,\$\$\$}$	$\downarrow^{\bullet,\$\$\$}$	$\downarrow^{***,\$\$}$	$\downarrow^{***,\$\$\$}$
Liq		$\downarrow^{*}$	$\uparrow^{**}$	-	$\uparrow^{***}$	$\uparrow^{***}$	$\downarrow^{**}$	$\downarrow^{***}$
MPP		$\downarrow^{*}$	$\uparrow^{**}$	-	-	$\uparrow^{***}$	$\downarrow^{***}$	$\downarrow^{***}$
Liq:MPP[d,f]	**1:1**	$\downarrow^{*,ns}$	$\uparrow^{***,ns}$	-	$\uparrow^{**,ns}$	$\uparrow^{***,ns}$	$\downarrow^{***,ns}$	$\downarrow^{***,\#\#\#}$
	10:1	nd	nd	nd	nd	$\uparrow^{*,ns}$	nd	nd
	100:1	nd	nd	nd	nd	$-^{ns}$	nd	nd
	1000:1	nd	nd	nd	nd	$-^{ns}$	nd	nd

(nd) = not done; (↑) = Increased; (↓) = Decreased; (-) = No significant effect

[a]Tested in the presence of 10^{-11} M E_2.

[b]Tested in the presence of 10^{-9} M E_2.

[c]Statistically different from solvent ($^{\bullet}$ represents $P < 0.05$, $^{\bullet\bullet}$ represents $P < 0.01$ and $^{\bullet\bullet\bullet}$ represents $P < 0.001$).

[d]Statistically different from E_2 (* represents $P < 0.05$, ** represents $P < 0.01$ and *** represents $P < 0.001$).

[e]Statistically different from 4-OH-Tam alone ($^{\$}$ represents $P < 0.05$, $^{\$\$}$ represents $P < 0.01$ and $^{\$\$\$}$ represents $P < 0.001$).

[f]Statistically different from SM6Met ($^{\#}$ represents $P < 0.05$, $^{\#\#}$ represents $P < 0.01$ and $^{\#\#\#}$ represents $P < 0.001$).

4.4 Discussion

In the process of cancer initiation a cancer cell acquires certain capabilities, which involves the ability to sustain cell growth signalling, evade inhibitory growth signals, avoid apoptosis, induce limitless replication, stimulate angiogenesis and activate the metastatic cascade (migration, invasion and colony formation) (4).

In chapter 3, the results from the MCF-7BUS proliferation assay showed that SM6Met was able to significantly inhibit E_2 induced breast cancer cell proliferation, albeit with a lower potency and efficacy than the SOC therapies. Furthermore, I demonstrated that the effects of SM6Met could be replicated by combining an ERα antagonist (MPP) with an ERβ agonist (liquiritigenin) and that in combination with 4-OH-Tam, SM6Met synergistically enhanced the effects of 4-OH-Tam to inhibit E_2 induced breast cancer cell proliferation.

Chapter 4 focused on: (1) how SM6Met compares to SOC therapies, like 4-OH-Tam and fulvestrant, (2) if the properties of SM6Met could be replicated by combining an ERα antagonist (MPP) with an ERβ agonist (liquiritigenin), and (3) how the synergistic combination of 4-OH-Tam with SM6Met compares to 4-OH-Tam alone, with regard to their effects on human breast cancer cell cycle regulation as well as metastatic potential. Results are summarized in Table 7.

4.4.1 Effects on breast cancer cell cycle

Cell proliferation relies on the controlled progression of cells through the cell cycle, a process dysregulated in cancer cells to gain infinite replicative potential, a hallmark characteristic of cancer (3, 4). Cell cycle analysis, a test system regarded as more accurate than proliferation studies, provides information on how a drug elicits its effects on cell proliferation with regard to cell cycle arrest and apoptosis (47, 48).

4.4.1.1 SM6Met, like liquiritigenin, induced apoptosis and the accumulation of MCF-7BUS cells in the S phase of the cell cycle

Various studies have shown that E_2 decreases the number of cells in the G0/G1 phase and increases the number of cells in the S phase and G2/M phase (49–51). However, in the current study E_2 (at the concentration 10^{-11}M) only slightly, but not significantly, increased the number of cells in the S phase (DNA synthesis).

In the presence of 10^{-11}M E_2, treatment with fulvestrant displayed similar cell cycle distribution patterns to previous studies where MCF7-BUS cells were treated with fulvestrant alone (51). Specifically fulvestrant caused an accumulation of cells in the G0/G1 phase (Fig. 4.6C), with no activation of apoptosis (Fig. 4.6F), thereby, suggesting that cell cycle progression was “paused” at the G0/G1 restriction checkpoint with cells entering a state of quiescence (rest) after treatment with fulvestrant. This restriction point is largely

controlled by the Rb/E2F signalling pathway, therefore, mechanistically fulvestrant could be eliciting its effects through upstream regulators of Rb, for example Cyclin D1, CDK4 or CDK6 (7). Previous studies showed that treatment with 4-OH-Tam alone induced a significant G0/G1 phase arrest (52–55), whereas in the current study, 4-OH-Tam, in the presence of 10^{-11}M E_2, induced a slight (not significant) increase in the number of cells in the S phase and in the number of apoptotic cells, while slightly decreasing the number of cells in the G2/M phase. However, taking into consideration the much higher concentrations of 4-OH-Tam used in previous studies (10^{-3}M – 10^{-6}M), 4-OH-Tam in the current study at the concentration of 10^{-9}M, most probably, had little to no effect on the cell cycle distribution.

In this study, SM6Met, in the presence of 10^{-11}M E_2, also displayed similar cell cycle distribution patterns to previous studies where MCF7-BUS cells were treated SM6Met alone (26). Specifically, SM6Met induced significant accumulation of cells in the S phase (Fig. 4.6D) as well as significantly increasing the number of apoptotic cells (Fig. 4.6F). It has previously been postulated that SM6Met may elicit its effects on the S phase by down-regulating cyclin-dependent kinase 2 (CDK2), thereby inhibiting DNA replication or by up-regulating inhibitors of CDK 2, like ataxia telangiectasia mutated (ATM) (56). MPP and liquiritigenin, like SM6Met, induced significant accumulation of cells in the S phase (Fig. 4.6D), while only liquiritigenin, like SM6Met, induced a significant number of apoptotic cells (Fig. 4.6F), suggesting that MPP and liquiritigenin alone, like SM6Met, inhibits cell proliferation via S phase arrest. No literature was found on the effects of MPP and liquiritigenin on breast cancer cell cycle distribution and thus comparison with literature is not possible.

In summary, SM6Met alone disrupts cell cycle progression via arresting cells in the S phase, while the SOC therapies, 4-OH-Tam and fulvestrant, arrests cells in the G0/G1 phase. This implies that SM6Met elicits its effects on the regulation of cell cycle machinery via a different mechanism than that of the SOC therapies, which is preferred for combination therapies as studies suggest breast cancer is more responsive to combinations that inhibit multiple molecular targets associated with the development and progression of breast cancer (57, 58). Therefore, SM6Met has great potential to be used in combination with current SOC therapies to provide a multi-targeted approach.

4.4.1.2 The effects of SM6Met on cell cycle progression could be replicated by combining an ERα selective antagonist (MPP) with an ERβ selective agonist (liquiritigenin)

The addition of 10^{-9}M liquiritigenin to 10^{-6}M MPP at a combination ratio of 1:1 resulted in a cell cycle distribution similar to that of SM6Met (Fig. 4.7). Specifically, although MPP and liquiritigenin on their own induced S phase accumulation, combining the two compounds resulted in a further significant increase in the number of cells in this phase to a level similar to that of SM6Met (Fig. 4.7D). Conversely, the effects of MPP and liquiritigenin on apoptosis seem to counteract each other as the combination significantly reduced the number of apoptotic cells relative to liquiritigenin, while increasing the number of apoptotic cells relative to MPP, however, the net result of the combination of liquiritigenin with MPP was similar to that of SM6Met.

Therefore I may postulate that the combination of MPP and liquiritigenin, like SM6Met, disrupts cell cycle progression via arresting cells in the S phase and inducing apoptosis.

To our knowledge, the combination of an ERβ selective agonist and an ERα selective antagonist and its effects on cell cycle regulation have not previously been investigated. However, these results support the notion that a compound with similar characteristics to the *Cyclopia* extract, SM6Met, could be synthesized to possibly avoid some of the limitations associated with natural compounds or extracts like low potency and effectiveness compared to synthetic drugs.

4.4.1.3 The addition of SM6Met to 4-OH-Tam not only increased the accumulation of cells in the S-phase, but also the number of cells in the apoptotic phase

The addition of SM6Met to 4-OH-Tam in the combination ratios 10:1 and 20:1 increased the number of cells in the S phase and the number of apoptotic cells, while decreasing the number of cells in the G2/M phase. This suggest that in combination SM6Met and 4-OH-Tam mechanistically enhanced S phase arrest, which conceivably lead to morphological changes and a subsequent increase in apoptosis. These findings show for the first time that in combination SM6Met enhances the pro-apoptotic effects of 4-OH-Tam. This implies that women taking tamoxifen as adjuvant therapy, may benefit further from co-treatment with SM6Met as a treatment strategy.

Similar to our study, a previous study by Charalambus *et al*. (59), showed that the combination of equol, the metabolite of the soy phytoestrogen, diazen, with 4-OH-Tam also significantly enhanced the number of apoptotic cells in comparison to equol and 4-OH-Tam alone through activation of caspase-mediated apoptotic pathways. To our knowledge there are no further studies on the effects of combinations of compounds or extracts with 4-OH-Tam on cell cycle progression. Thus, the mechanisms by which SM6Met and other compounds enhance the anti-breast cancer activity of tamoxifen are still largely unknown.

4.4.2 Effects on metastatic potential of breast cancer cells

Despite the advances in treatment targeting primary tumours, treatment strategies for metastasis have been less effective and development of secondary tumours play a major role in the high mortality still associated with cancer (60–62). Therefore, I looked at the effects of the test panel on hallmark capabilities of cancer cells that are involved in metastasis i.e. cell motility, cell invasion and anchorage independent growth.

4.4.2.1 SM6Met reduced E_2 induced breast cancer cell invasion and colony formation to a level comparable to that of the SOC therapies, but also like all the other test compounds induced breast cancer cell migration

Previous studies have shown that in ER-positive breast cancer cells E_2 stimulates migration through activation of mitogen-activated protein kinase (MAPK) phosphorylation of cSRC which in turn interacts with

focal adhesion kinases and the delta 5 truncated form of SRC3. This process stimulates the development of filopodia and pseudopodia at the leading edges of the breast cancer cells (63–65). However, in contrast to previous studies, I showed that 10^{-9}M E_2 inhibited breast cancer cell migration (66, 67), which may be attributed to differences in methodology like different cell lines, induction periods, culture conditions and the use of mytomycin C, an inhibitor of cell proliferation used to truly identify the migratory potential. As the use of mitomycin C has only recently been introduced to migratory studies (wound healing assays) to distinguish between actual migration and proliferation, no literature was found to corroborate the effects of the test panel on breast cancer cell migration. Nevertheless, all the test compounds and extract reversed the protective effect of E_2 on cell migration with MPP displaying the highest induction of migration, thus, suggesting a protective role for ERα against breast cancer cell migration and shows promise for therapeutic strategies that do not primarily target ERα for the treatment and prevention of breast cancer.

There is contradicting evidence as to the effects of E_2 on breast cancer cell invasion as some studies have shown that E_2 reduces breast cancer cell invasion (66), some show no effect of E_2 on breast cancer cell invasion (26, 68) and others, like our study, indicate an increase in breast cancer cell invasion (40, 68). To our knowledge this is the first time that the effects of 4-OH-Tam alone on breast cancer cell invasion were analysed and showed significant inhibition of invasion. Although 4-OH-Tam, in the presence of E_2, had no significant effect on breast cancer cell invasion in the current study, it has previously been shown that 4-OH-Tam increases breast cancer cell invasion in relation to E_2 (66, 68). In contrast to a study by Foty *et al.* (69), fulvestrant on its own in the current study reduced breast cancer cell invasion, whereas similar to previous studies, fulvestrant reduced E_2 induced breast cancer cell invasion in the current study (69). I show for the first time that SM6Met, in the presence and absence of E_2, was able to inhibit breast cancer cell invasion. SM6Met alone reduced invasion to a greater extent than the SOC therapies, 4-OH-Tam and fulvestrant, while in the presence of E_2, SM6Met displayed a similar level of inhibition as fulvestrant.

In relation to previous studies E_2 (at the concentration 10^{-11}M in the current study) displayed an increase in colony formation (70, 71). However, to our knowledge this is the first time that it has been shown that increasing the concentration of E_2 reduces colony formation in a dose dependent manner, suggesting a possible biphasic effect of E_2 on breast cancer colony formation. All the test compounds as well as the extract were able to inhibit E_2 induced colony formation, with the ERα selective ligand, MPP, displaying the highest level of inhibition and SM6Met showing similar inhibition levels to 4-OH-Tam and liquiritigenin. Except for 4-OH-Tam that has previously been shown to decrease E_2 induced colony formation (70), no previous studies were found to corroborate the findings of the current study. Nevertheless, these findings suggest that ERα plays a positive role in the induction of colony formation.

In summary, these results show that SM6Met is just as effective as the SOC therapies at targeting processes involved in breast cancer metastasis, like invasion and colony formation, and supports the

concept that by targeting and inhibiting one of the pro-metastatic processes like cell invasion through therapeutic interventions metastasis may be inhibited or delayed (72, 73).

4.4.2.2 The addition of liquiritigenin (ERβ selective agonist) to MPP (ERα selective antagonist) reduced migration and colony formation to levels exceeding that of SM6Met and the SOC therapies, 4-OH-Tam and fulvestrant

To our knowledge, the combination of an ERβ selective agonist and an ERα selective antagonist and its effects on the biological processes implicated in breast cancer metastases including cell migration, invasion and colony formation have not previously been investigated. Therefore, the results pertaining to the effects of this combination on breast cancer cell migration, invasion and colony formation are novel.

In the presence of E_2, the addition of liquiritigenin to MPP, reduced the high migratory effects of MPP, to a level lower than that of SM6Met alone and similar to the solvent treated cells, whereas the combination of MPP and liquiritigenin, in the presence and absence of E_2, inhibited breast cancer cell invasion to a degree comparable to that of SM6Met alone. Moreover, E_2 induced colony formation was inhibited to a greater extent than with SM6Met, liquiritigenin and MPP alone, when liquiritigenin was combined with MPP. However, the slight 2.96-fold increase in the inhibitory effect of MPP in the presence of liquiritigenin was not statistically different to that of MPP on its own. Taken together, in contrast to the proliferation studies (Ch. 3) were the combination of MPP with liquiritigenin mimicked the effects of SM6Met, the combination exerted effects greater than that of SM6Met on migration and colony formation, suggesting that a compound with the ability to strongly agonize ERβ, while antagonizing ERα, may regulate genes and proteins associated with these processes in such a way that it inhibits the development of migratory and anchorage independent characteristics of the cell. This also implies that a compound that may elicit the same beneficial characteristics of SM6Met with greater efficacy could be synthesized.

4.4.2.3 Combining SM6Met with tamoxifen in a ratio of 20:1 inhibited all three processes implicated in breast cancer metastasis to a degree greater than that of the rest of the test panel

The results of this study show, for the first time, that SM6Met in combination with 4-OH-Tam in a ratio of 20:1 could inhibit breast cancer cell migration to a level greater than that of SM6Met or 4-OH-Tam alone and similar to that of 10^{-9}M E_2, the compound with the highest inhibitory effect on migration in the current study. Furthermore, in the presence of E_2, SM6Met alone inhibited breast cancer cell invasion to a similar extent as fulvestrant and to a greater extent than 4-OH-Tam. However, the addition of SM6Met to 4-OH-Tam, decreased the number of invasive breast cancer cells further than SM6Met or 4-OH-Tam alone, in a dose dependant manner. The ERα selective antagonist, MPP, displayed the highest level of inhibition of colony formation, while SM6Met displayed similar inhibition levels as 4-OH-Tam and liquiritigenin, but less than fulvestrant alone. However, when adding SM6Met in combination with 4-OH-Tam, enhanced inhibition of colony formation to a level similar to that of MPP was observed. Hence, the combination of SM6Met with

4-OH-Tam was more effective at targeting breast cancer cell migration, invasion and colony formation than the SOC therapies, 4-OH-Tam and fulvestrant, alone. As the combination of SM6Met and 4-OH-Tam was the only treatment to substantially inhibit all three processes implicated in breast cancer metastasis (Table 1), it shows great potential to not only be developed as treatment for primary or early stage breast cancer, but also metastatic breast cancer.

4.4.3 Role of ER signalling in breast cancer progression and metastasis

Although this study focuses purely on the physiological effects of the test panel on processes involved in breast cancer progression and metastasis i.e. proliferation, migration, invasion and colony formation, the results from this study provide some insight into the role of the ER subtypes, ERα and ERβ, on breast cancer progression and metastasis.

With regard to cell cycle regulation in the presence of E_2, ERα has been implicated in various cell cycle regulatory events that promote cell proliferation, including positive regulation of cyclin D1 and A and inhibition of p27 nuclear accumulation (74, 75). Although ERβ has not been as extensively studied as ERα, it has been shown to counteract the proliferative effects of ERα, by negatively regulating cyclin D1 and activating p21 and p27 expression (76, 77). Unfortunately, due to time and financial constraints I were unable to validate the effects of the test panel on cell cycle distribution in the presence of E_2. However, this study supports previous findings on the physiological role of the ERs, characterizing ERα as mediator and driving component of breast cancer cell proliferation and supports the anti-proliferative effects of ERβ.

Furthermore, early studies on cancer, including breast cancer, suggested that ERα signalling inhibits migration and invasion (78). However, more recent studies have contradicted these findings and found that ERα signalling promotes motility and invasiveness (79–83) and thereby drives metastasis. In contrast, ERβ expression has been associated with less invasive and migratory cells (84–87). With regard to breast cancer cell migration, the results of the current study showing that E_2 inhibits breast cancer cell migration, while MPP, an ERα antagonist, counteracts the effects of E_2, support earlier studies suggesting that ERα signalling plays a protective role in breast cancer cell migration. Conversely, similar to other recent studies (79–83), the results of the current study implicate ERα activity in the promotion of breast cancer invasiveness. Moreover, our study supports the anti-migratory role of ERβ, as the results of the current study show that by increasing the concentration of liquiritigenin, an ERβ selective agonist, in combination with MPP, the high migratory effect of MPP is reduced to the same effect level as that of the solvent treated cells.

No previous literature was found on the possible role of the ER subtypes, ERα and ERβ, in breast cancer colony formation. Moreover, except for 4-OH-Tam that has been shown to decrease E_2 induced colony formation (70), no previous studies were found to support our findings of the effects of the other test compounds and the SM6Met extract in the presence of E_2 on breast cancer colony formation. Despite this

the overwhelming inhibitory effect of MPP on colony formation in the current study would suggest a role for ERα. Collectively, the results of the current study implicate ERα in the promotion of breast cancer cell proliferation, invasion and colony formation, while suggesting an anti-migratory role for ERα, and implicating ERβ in the inhibition of breast cancer cell proliferation, migration, invasion and colony formation.

4.4.4 Conclusion

The results of this study present insights into the potential of SM6Met as a treatment for metastatic breast cancer, either as monotherapy or in combination with current SOC therapies, like 4-OH-Tam. It is important to remember that this study only investigated the combinations of the IC_{50} ratios and that other concentration combinations could yield more beneficial results in terms of reducing breast cancer metastasis. As monotherapy, SM6Met was able to inhibit two out of the three pro-metastatic processes evaluated in this study (Table 1). Specifically, SM6Met was just as effective or more effective at inhibiting E_2 induced breast cancer cell invasion and colony formation as fulvestrant and 4-OH-Tam. Thus, SM6Met shows promise as an inhibitor of metastasis by targeting pro-metastatic processes.

However, the most exciting outcomes of this study are the results pertaining to the combination therapies. Firstly, although the 1:1 combination ratio of liquiritigenin with MPP, like SM6Met, induced breast cancer cell migration, by increasing the concentration of liquiritigenin in the combination ratio with MPP to 1000:1, the high migratory effect of MPP was reduced to the same level as that of the solvent treated cells. This suggests that combining an ERβ selective agonist with an ERα selective antagonist may provide a novel means of targeting breast cancer metastasis and highlighting the therapeutic potential of ER subtype selective modulators, otherwise known as selective estrogen receptor subtype modulators (SERSMs). Furthermore, the in terms of invasion and colony formation the 1:1 ratio of liquiritigenin and MPP was generally more effective than the compounds alone.

Secondly, the combination of SM6Met with 4-OH-Tam at a ratio of 20:1 was the only treatment able to substantially inhibit all three pro-metastatic processes evaluated in this study i.e. migration, invasion and colony formation in the presence of E_2. Thus, combined therapies with a compound or extract with ERβ agonist and ERα antagonist properties such as SM6Met may provide a novel approach for the treatment and or prevention of metastatic breast cancer. These promising effects warrant further investigation into the mechanisms through which SM6Met enhances the effects of 4-OH-Tam. Furthermore, once the mechanism of action is established, one may predict and study the possible synergistic effects of SM6Met in combination with other SOC therapies.

4.5 Literature cited

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Chapter 5

Final discussion and conclusions

Breast cancer is globally the most commonly diagnosed cancer amongst women (23% of all cancer cases) and is responsible for 14% of all cancer deaths (1). Estrogen overexposure has been shown to play a major role in breast cancer development and progression through induction of hyper-proliferation and generation of genotoxic metabolites, which inflict DNA damage (2–4). Therefore, current hormone treatments focus on blocking the estrogen signalling pathway. For example, AIs or ovarian function suppressors target estrogen synthesis (5–10), while SERMs and SERDs target estrogen receptors (ERs) (11–15). However, blocking estrogen signalling can induce menopause-like side-effects (11–19) as well as increase the risk of inflammatory diseases (20, 21) and osteoporosis (10, 19, 22). Furthermore, despite the overall effectiveness of these endocrine therapies, some breast cancer cases develop resistance (23–25), resulting in the need for the development of an alternative approach to breast cancer treatment with lower side-effect profiles and a reduced risk of resistance development.

Previous studies have characterized ERα, especially in breast cancer cells, as mediator and driving component of cell proliferation in the presence of estradiol (26), while ERβ has shown anti-proliferative effects on breast cancer cells by opposing the actions of ERα (27, 28). The physiological function of the two ER subtypes suggest that an agent with the dual effect of antagonising ERα, while activating ERβ may provide a safer, more effective alternative to prevent breast cancer initiation and/or inhibit breast cancer progression, which can either be used as monotherapy or in combination with existing therapies.

SM6Met, an extract from the fynbos plant *Cyclopia subternata* most commonly known as honeybush, has previously been shown to act as an ERα antagonist and ERβ agonist (29), to have anti-inflammatory effects and to inhibit E_2 induced breast cancer cell proliferation, uterine- and mammary tumour growth (30, 31). Therefore, the main aim of this study was to explore the physiological implications of the dual activity (multi-targeting) of the SERSM, SM6Met, on the processes required for the development and progression of breast cancer such as proliferation, migration, invasion and colony formation, either as monotherapy or in combination with current SOC therapies, like tamoxifen. The results of the current study will be discussed in terms of three research questions.

5.1 How does SM6Met, as monotherapy, compare to SOC therapies, like 4-OH-Tam and fulvestrant, with regard to their effects on breast cancer cell proliferation, migration, invasion and colony formation?

To answer the first research question (Fig. 5.1), the MCF7-BUS cell line was used to analyse the effects of the test panel as it represents an integrated model where both ER subtypes are co-expressed and contribute to the final phenotype (20, 21, 32). From this test system it was found that although SM6Met, like

4-OH-Tam and fulvestrant, was able to significantly inhibit E_2 induced breast cancer cell proliferation, it could not attain the same potency nor efficacy as the SOC therapies, 4-OH-Tam and fulvestrant. Similarly, SM6Met was able to significantly inhibit breast cancer cell invasion and colony formation. However, with regard to efficacy, SM6Met, in the presence of E_2, displayed a similar efficacy as fulvestrant at inhibiting cell invasion, while 4-OH-Tam had no effect. Furthermore, although fulvestrant showed greater inhibition of colony formation, in the presence of E_2, than SM6Met, SM6Met displayed similar efficacy to that of 4-OH-Tam. Interestingly, the whole test panel induced breast cancer cell migration, with SM6Met displaying similar induction levels as the SOC therapies, 4-OH-Tam and fulvestrant. Together these results suggest that SM6Met as monotherapy cannot compete with current SOC therapies at targeting breast cancer cell proliferation, however, SM6Met may prove just as effective as the SOC therapies at targeting pro-metastatic processes including breast cancer cell invasion and colony formation. Therefore, SM6Met shows great promise to be developed into a nutraceutical for the treatment or prevention of breast cancer metastasis.

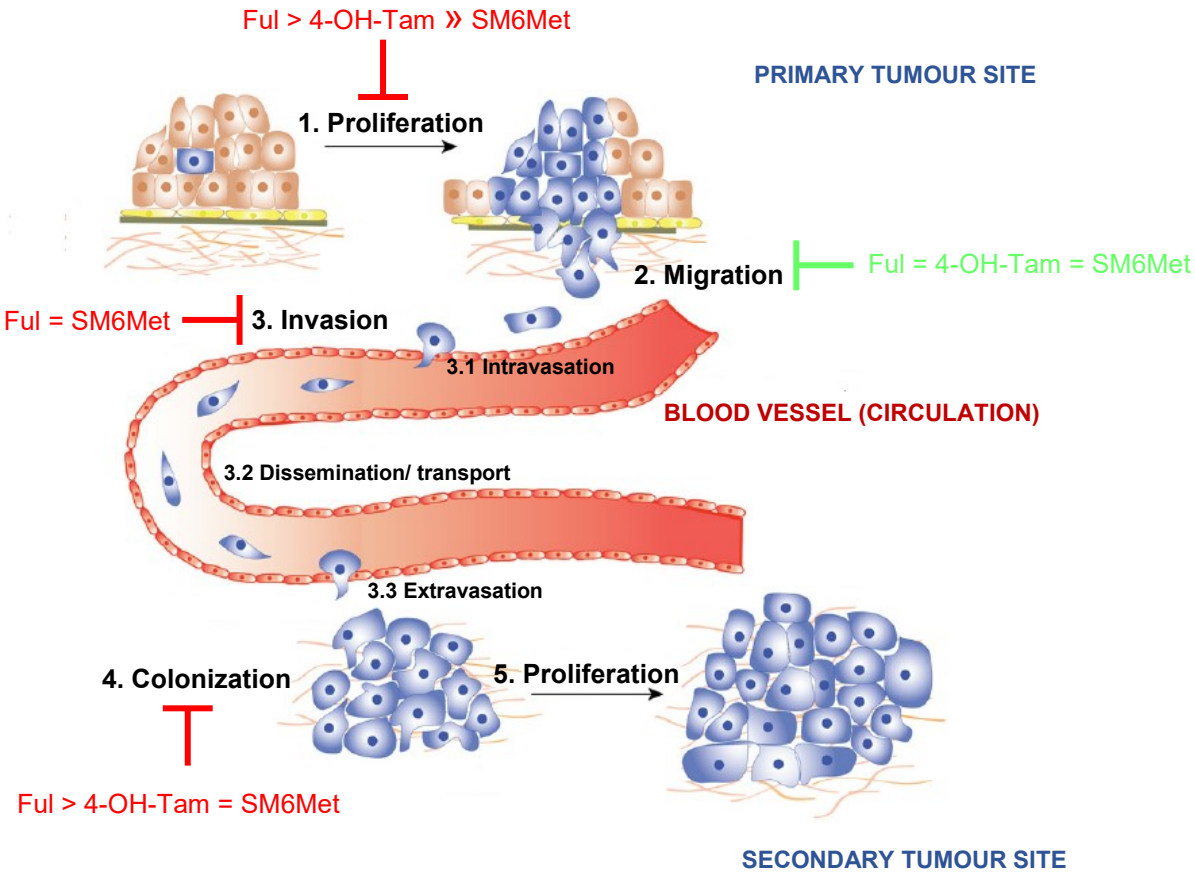
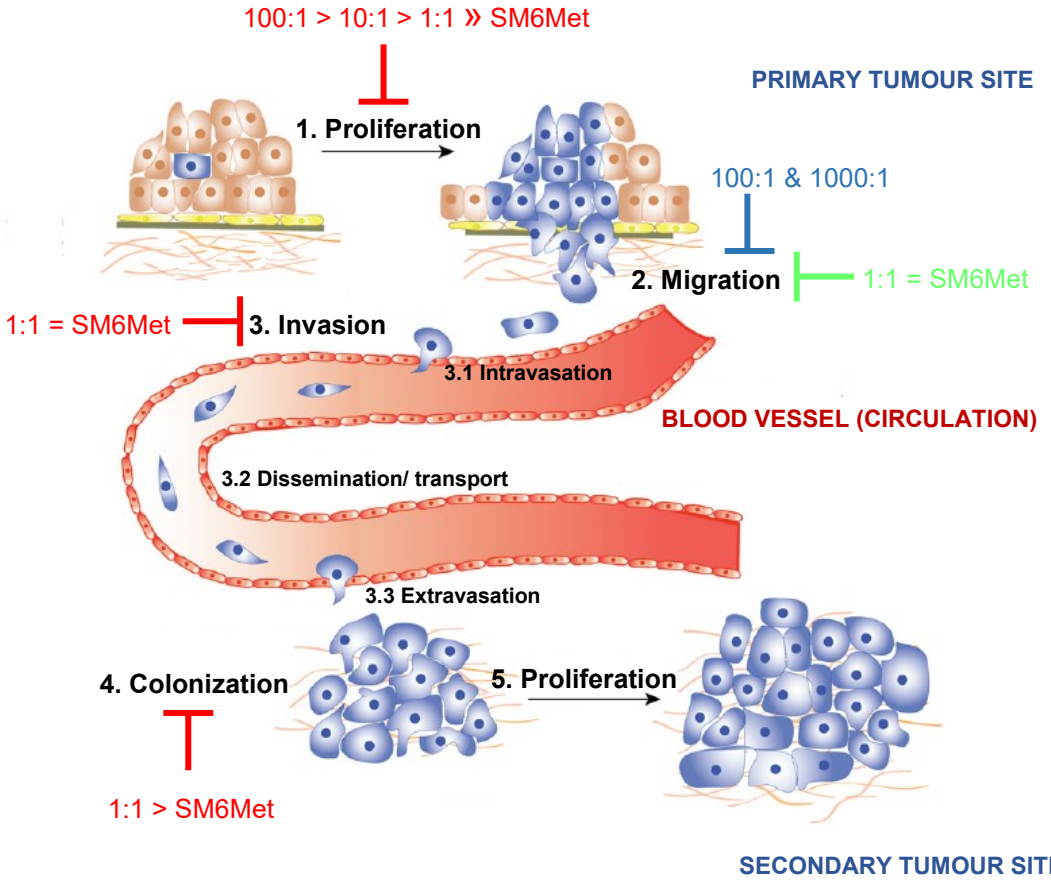


Figure 5.1: Schematic representation of the effects of SM6Met in comparison to the SOC therapies, 4-OH-Tam and fulvestrant, on the processes involved in breast cancer development and progression. Breast cancer carcinogenesis encompasses various steps, each of which present an opportunity for new therapies. Carcinogenesis is characterized by uncontrolled cell proliferation (1), which leads to the acquisition of specific properties which allow the tumour cell to detach, migrate (2) and invade (3) local tissue to ultimately enter into circulation, travel to distant organs and form colonies (4) at the secondary tumour site. Here the red solid line indicates the inhibiting effects of SM6Met, fulvestrant and 4-OH-Tam in order of efficacy, while the green solid line represents induction. Figure adapted from Saxena and Christofori (33).

5.2 Can the SERSM properties of SM6Met be replicated by the combination of an ERα antagonist (MPP) and an ERβ agonist (liquiritigenin)?

Natural extracts are commonly associated with lower potency and efficacy in comparison to synthetic compounds. Therefore, the active constituent is usually characterized, isolated, chemically replicated and synthesized. However, a recent study showed that the desirable estrogenic effects of SM6Met could not be retained or significantly enhanced by fractionation (34). This raised our second research question (Fig. 5.2): that if the active constituent in SM6Met could not be identified or isolated, could the SERSM effects of SM6Met be replicated by combining an ERα antagonist (MPP) with an ERβ agonist (liquiritigenin). Despite several studies and reviews advocating the approach (35–40), to our knowledge the combination of a selective ERα antagonist, like MPP, with a selective ERβ agonist, like liquiritigenin, has not previously been investigated for breast cancer treatment or prevention and all results pertaining to the effects of this combination are novel. The most effective combination (10^{-7}M liquiritigenin together with 10^{-6}M MPP also referred to as 100:1) of these compounds was not only more effective at inhibiting E_2 induced breast cancer cell proliferation than 10^{-6}M MPP alone and 10^{-7}M liquiritigenin alone, but was also more effective than SM6Met and the SOC therapies, 4-OH-Tam and fulvestrant. Furthermore, although the combination of MPP with liquiritigenin at ratio 1:1 (10^{-9}M liquiritigenin together with 10^{-6}M MPP) induced breast cancer cell migration, in the presence of E_2, to a slightly greater, yet not significant extent than SM6Met, by increasing the concentration of liquiritigenin up to a ratio of 20:1 and 30:1 in combination with MPP, the high migratory effects of MPP and liquiritigenin were reduced to a level similar to solvent treated cells and substantially below that of SM6Met. The 1:1 combination of liquiritigenin with MPP inhibited breast cancer cell invasion to a similar extent as SM6Met and to a greater extent than the compounds alone, while this combination displayed greater inhibition of colony formation, in the presence of E_2, than SM6Met or than the compounds alone. Together these results suggest that combining an ERα antagonist (MPP) with an ERβ agonist (liquiritigenin) improves the anti-proliferative and anti-metastatic potential of the individual compounds; that the ideal ER subtype selective properties of SM6Met can be replicated or enhanced; and that a purer, highly concentrated synthetic drug or nutraceutical combination with the same SERSM characteristics could possibly be produced.



1000:1 = 10^{-6}M Liq + 10^{-6}M MPP; 100:1 = 10^{-7}M Liq + 10^{-6}M MPP; 10:1 = 10^{-8}M Liq + 10^{-6}M MPP; 1:1 = 10^{-9}M Liq + 10^{-6}M MPP

Figure 5.2: Schematic representation of the effects of the combination of liquiritigenin with MPP compared to SM6Met on the processes involved in breast cancer development and progression. Breast cancer carcinogenesis encompasses various steps, each of which present an opportunity for new therapies. Carcinogenesis is characterized by uncontrolled cell proliferation (1), which leads to the acquisition of specific properties which allow the tumour cell to detach, migrate (2) and invade (3) local tissue to ultimately enter into circulation, travel to distant organs and form colonies (4) at the secondary tumour site. Here the red solid line indicates the inhibiting effects of the combinations of liquiritigenin with MPP and SM6Met in order of efficacy, while the green solid line represents induction and the blue line represents no effect. Figure adapted from Saxena and Christofori (33).

5.3 Does SM6Met act synergistically in combination with 4-OH-Tam?

The recent development of combined therapies, many of which notably include the use of more natural products, such as tea leaf extracts, in combination with conventional chemotherapeutic agents have been driven by the drawbacks associated with current SOC therapies, like severe side-effect profiles and the occurrence of endocrine resistance (41–43). As many of these combinations have been shown to produce synergistic activity or enhancement of the anti-cancer effects of tamoxifen (42, 43), our third and final research question (Fig. 5.3) entailed the investigation of the potential synergistic effects of SM6Met in combination with 4-OH-Tam and how the potential synergistic combination of 4-OH-Tam with

SM6Met compares to 4-OH-Tam alone. For the first time the current study demonstrated that the combination of 4-OH-Tam (the active metabolite of tamoxifen) and SM6Met (a honeybush extract) produces a strong synergistic effect in terms of antagonising E_2 induced breast cancer cell proliferation. In combination with SM6Met, 20 times lower concentrations of 4-OH-Tam are required to produce the same inhibitory effect on cell proliferation as with 4-OH-Tam alone.

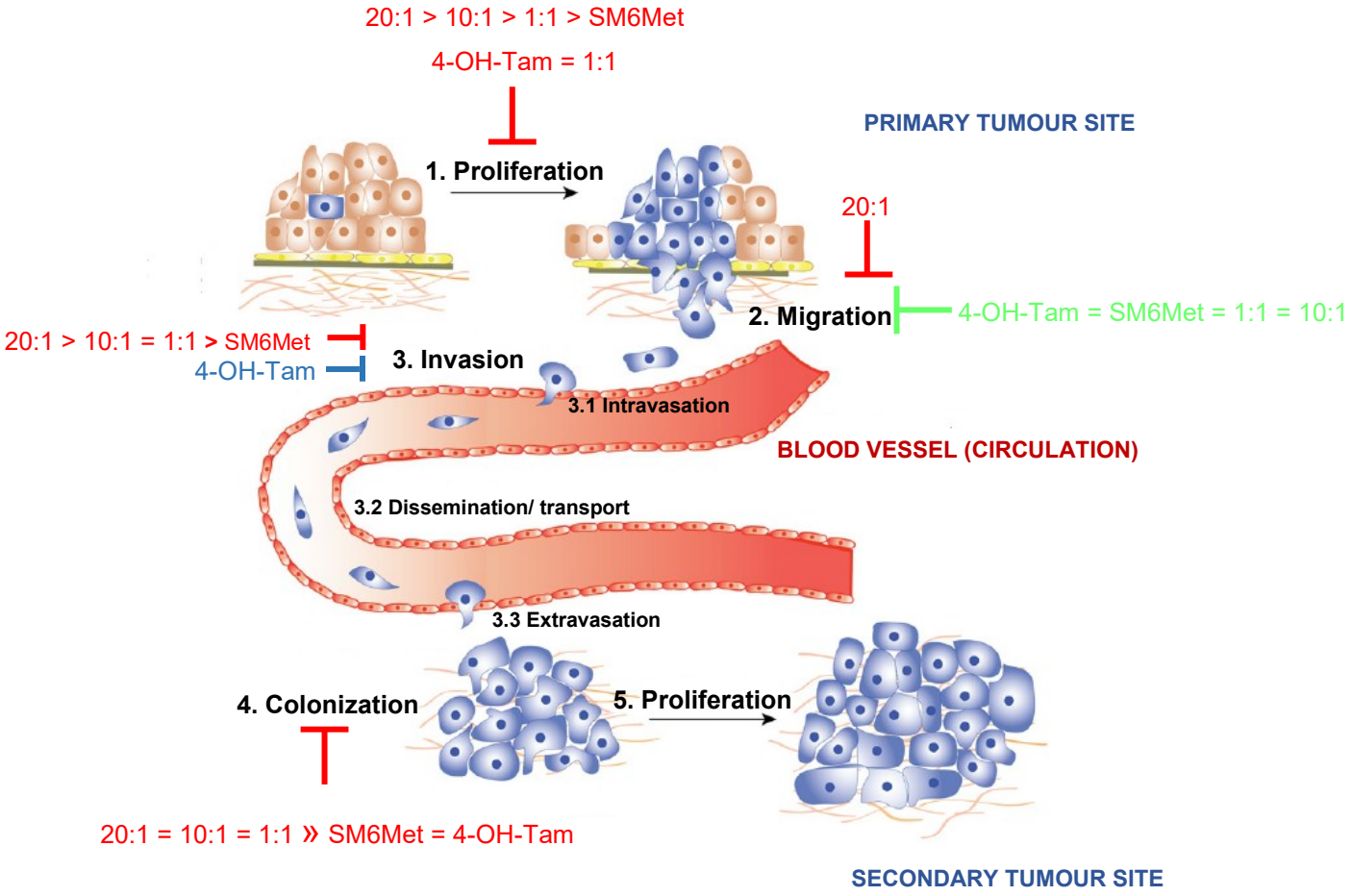


Figure 5.3: Schematic representation of the effects of the combinations of SM6Met with 4-OH-Tam compared to SM6Met alone and 4-OH-Tam alone on the processes involved in breast cancer development and progression. Breast cancer carcinogenesis encompasses various steps, each of which present an opportunity for new therapies. Carcinogenesis is characterized by uncontrolled cell proliferation (1), which leads to the acquisition of specific properties which allow the tumour cell to detach, migrate (2) and invade (3) local tissue to ultimately enter into circulation, travel to distant organs and form colonies (4) at the secondary tumour site. Here the red solid line indicates the inhibiting effects of the combinations of SM6Met with 4-OH-Tam, SM6Met alone and 4-OH-Tam alone in order of efficacy, while the green solid line represents induction and the blue represents no effect. Figure adapted from Saxena and Christofori (33).

Furthermore, increasing the concentration of SM6Met in combination with 4-OH-Tam to a ratio of 20:1 resulted in an overall inhibition of breast cancer cell migration not seen with either 4-OH-Tam or SM6Met alone. The combination of SM6Met with 4-OH-Tam was the only treatment strategy, apart from E_2, to inhibit breast cancer cell migration. Although 4-OH-Tam, in the presence of E_2, had no significant effect on breast

cancer cell invasion, when added in combination with SM6Met it displayed significant inhibition to a level greater than that of SM6Met. Specifically, the 20:1 combination ratio of SM6Met with 4-OH-Tam displayed the highest inhibition of breast cancer cell invasion compared to the rest of the test panel and other combination mixtures, including that of MPP and liquiritigenin. Moreover this combination of SM6Met with 4-OH-Tam was able to significantly inhibit breast cancer colony formation to the same extent as MPP, which displayed the highest level of inhibition and to a significantly greater extent than either 4-OH-Tam or SM6Met alone. Therefore, these results suggest that SM6Met with 4-OH-Tam could be a viable drug combination, which may potentially delay resistance and ameliorate the negative side effects associated with tamoxifen monotherapy on breast cancer patients and may suppress the migratory, invasive and anchorage independent nature of MCF-7 cells and may ultimately inhibit or prevent metastatic progression of breast cancer. In addition the lower dose of tamoxifen and incorporation of a honeybush extract do suggest that this would also be a more affordable alternative to conventional chemotherapy and highlights the potential of honeybush tea to be used as a dietary intervention for the prevention of breast cancer.

5.4 Conclusion

To conclude, the well-characterized phytoestrogenic extract, SM6Met, has previously been shown to have an estrogenic potency comparable to commercially available phytoestrogenic nutraceuticals (29, 44), to display subtype selective activity by acting as an ERα antagonist and ERβ agonist (29), to inhibit E_2 induced breast cancer cell proliferation through G0/G1 arrest like tamoxifen, to induce S phase arrest in the absence of E_2 (45), to antagonize E_2 induced uterine growth and delay vaginal opening in an immature rat uterotrophic model (29) as well as inhibit rat mammary tumour growth (30, 31). However, none of these studies investigated the metastatic potential of SM6Met nor the combination of SM6Met with a conventional chemotherapeutic or endocrine therapy agent.

The key findings of the current work lie in the following. Firstly, with regard to SM6Met as monotherapy, although SM6Met could not compete with the efficacy or potency of SOC treatments, like 4-OH-Tam and fulvestrant, at targeting breast cancer cell proliferation, SM6Met showed promising anti-metastatic potential equal if not better than the SOC therapies by targeting two components of the metastatic pathway, invasion and colony formation. Secondly, for the first time the combination of an ERβ selective agonist (liquiritigenin) and an ERα selective antagonist (MPP) was evaluated and found to not only inhibit breast cancer proliferation to a degree greater than the rest of the test panel, but also to display promising anti-metastatic potential greater than that of current SOC therapies by not inducing breast cancer cell migration and by targeting invasion and colony formation, thereby validating the concept that an ideal SERSM would antagonize ERα, while activating ERβ and may be more beneficial for the treatment and prevention of breast cancer. Thirdly, for the first time the combination of SM6Met with 4-OH-Tam was proven to be synergistic in inhibiting breast cancer cell proliferation, while the best combination ratio (20:1) showed the highest degree of anti-metastatic potential as it was the only treatment to inhibit all three components of the

metastatic pathway that was evaluated in this study i.e. migration, invasion and colony formation, suggesting that combination therapy is not only a viable strategy for delaying resistance and ameliorating the negative side effects associated with current SOC endocrine therapies, like tamoxifen, but could also provide a new therapeutic strategy for preventing breast cancer metastasis. In addition the lower dose of tamoxifen required when combined with SM6Met, a plant extract, suggests that this would also be a more affordable alternative to conventional chemotherapy.

It is clear that plants extracts have great potential in combination therapies, as strategy to overcome the drawbacks of resistance and severe side-effects associated with current SOC endocrine therapies. However, future work is needed to not only elucidate the molecular mechanism of SM6Met, but to also validate the current findings and establish the optimal combination ratios of the mixtures evaluated in this study, especially as pertaining to metastatic potential.

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